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(54) Title: GENE CONFERRING SALT TOLERANCE

(57) Abstract

A single gene can confer sodium and/or lithium tolerance upon a cell. For example, the sod2 gene isolatable from *S. pombe* confers sodium and/or lithium tolerance upon cells such as yeast and plants cells. The gene can be used to produce salt tolerant yeasts, plants and other organisms.

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GENE CONFERRING SALT TOLERANCE

Background

Sodium chloride is the most abundant salt and generally the major component contributing to salinization resulting from irrigation of soil. Another sodium salt, sodium sulphate, is also a major contributor to salinization, particularly in western North America. Other soluble salts can "poison" soil but are less commonly encountered.

The principal symptoms of salt "poisoning" are gradual lowering of crop yield and vitality; ultimately, plants will neither germinate nor grow. In its early stages, it may not be noticed by farmers since, in general, up to twenty percent reduction in yield may not be apparent under field conditions.

Salts are ionic materials and in general the cation is the entity actively transported by physiological pumps. In the case of sodium chloride, chloride ions simply follow sodium ions to maintain electrical charge balance. Sodium transport mechanisms govern completely. There are two types of transport involved: active (or pumping) and passive (or diffusion). Plants are designed to acquire potassium and to reject or export sodium. Both acquisition and export of ions require active pumps. Passive transport is driven by concentration gradients. Active transport requires coupling to a metabolic energy source.

Certain plants and yeasts have been identified as being more "salt-tolerant" than others. There are a wide variety of physiological mechanisms which

contribute to tolerance at the level of the intact plant including salt excretion and transport within the plant. Extreme halophytes have an extensively modified cytoplasmic physiology to deal with high internal salt concentrations; this mechanism may not be suitable for creating salt tolerant cultivars.

Degrees of salt tolerance are shown by certain crops such as barley but in all cases yield penalties rise with salt stress. Despite years of study, no single gene or gene product directly involved in sodium (or chloride) transport or tolerance in plants has been identified. It has been thought that a cluster of genes all acting "simultaneously" is required. Identification and characterization of such genes would be of great biological, agricultural and industrial importance.

Summary of the Invention

This invention pertains to a gene which confers salt (sodium and/or lithium ion) tolerance upon a cell, to methods of conferring salt tolerance upon cells and to salt tolerant cells and organisms. The invention is based in part on the discovery that a single gene can transform a cell to sodium and lithium tolerance. The sod2 gene, isolatable from the Schizosaccharomyces pombe, can transform cells such as yeast and plant cells to sodium and lithium tolerance. The gene is itself sufficient, when either overexpressed or introduced into a cell in a sufficient number of copies, to transform a cell to a sodium tolerant phenotype. The gene can be used to create salt tolerant varieties of yeast, plants and other organisms.

Brief Description of the Drawings

Figures 1A, B & C illustrate the pH dependence of cation tolerance of wild type Schizosaccharomyces pombe.

Figure 2 illustrates the relative growth rate of wild type and sod2-1 S. pombe cells on agar plates at high NaCl concentrations.

Figure 3A, B & C illustrate the growth rate of wild type, sod2-1 and psod2 ura4-D18 S. pombe cells in liquid culture relative to NaCl concentration.

Figures 4A, B & C illustrate the growth rate of wild type, sod2-1 and psod2 ura4-D18 S. pombe cells in liquid culture relative to Na_2SO_4 concentration.

Figure 5 illustrates sodium uptake in wild type S. pombe cells.

Figure 6 illustrates sodium uptake in sod2-1 S. pombe cells.

Figure 7 illustrates sodium export from wild type and sod2-1 S. pombe cells.

Figure 8 illustrates the plasmid map of psod2.

Figure 9 illustrates the coding region and deletion map of the sod2 gene.

Figure 10 is the nucleotide and encoded amino acid sequence of the sod2 gene.

Figure 11A is a Southern blot analysis of the sod2 gene in wild type S. pombe cells using the 5.8 kb genomic insert of psod2 as probe.

Figure 11B is a Southern blot analysis of the sod2 gene in sod2-1 cells probed as in 11A.

Figure 11C is a Southern blot analysis of the sod2 gene in sod2-1 cells using the 2.3 kb HindIII genomic insert of psod2 as probe.

Figure 12 is a Southern blot analysis of genomic DNA from sod2::ura4.

Figure 13 is a plasmid map of psod2-ADH1.

Figure 14 shows the growth of wild type, sod2-1, psod2-ADH1 and psod2-ADH2 strains on agar plates containing LiCl.

Figure 15 illustrates sodium export in the absence of external sodium for wild type, sod2-1, psod2-ADH1 leu1-32 and sod2::ura4.

Figure 16 is the same as Figure 15 but with external sodium.

Figure 17 is a depiction of the plasmid pRMI containing a sod2 cDNA insert.

Figure 18 shows the plasmid pCGS110-sod2 containing the sod2 gene under control of the galactose-inducible promoter Gal1 used for transformation of S. cerevisiae.

Figure 19 shows the lithium tolerance of S. cerevisiae grown with galactose as the carbon source and rescue by p CGS110-sod2.

Figure 20 shows the growth of S. cerevisiae transformed with pCGS110-sod2 at high lithium concentration with galactose as a carbon source.

Detailed Description of the Invention

The gene of this invention confers salt tolerance in cells. As used herein, the trait of salt tolerance denotes the increased ability of a cell (transformed with the gene) to survive at a particular sodium and/or lithium concentration as compared with a wild type cell (untransformed). In the case of higher organisms, such as plants, the salt tolerant phenotype can be manifested as an increased survivability or productivity of the transformed variety at a particular sodium or lithium concentration over the wild type or untransformed plant.

The preferred embodiment of the gene conferring salt tolerance has the nucleotide sequence (genomic) shown in the Sequence Listing (and in Figure 10). The gene is designated sod2 and is isolatable from the yeast S. pombe. As described in detail below, the gene confers salt tolerance upon cells such as yeast and plant cells. The gene, expressed at appropriate levels, is itself sufficient to confer a salt tolerant phenotype upon a cell.

This invention embraces any nucleic acid sequence which, as a single gene (i.e., a gene which encodes a single protein or polypeptide product), can confer salt tolerance upon a cell. This includes nucleic acids having sequences identical to the sod2 sequence or sufficiently complementary to the sequence to be active in conferring salt tolerance and any transcripts of the sod2 DNA sequence or its variants. It also includes any nucleotide sequence which encodes the amino acid sequence given in the Sequence Listing.

As would be clear to a person of ordinary skill in the art, the nucleotide sequence of sod2 given in the Sequence Listing can be changed without necessarily affecting gene activity. For example, additions, deletions, insertions or substitutions of nucleotides in the sequence can be made. The variant forms may have equivalent or improved activity or may be designed to conform to the codon usage of the cell type to be transformed. In addition, active fragments of the gene may be identified. The term sod2 is inclusive of all variant, salt-tolerance-conferring forms of this gene.

Additional variants of the sod2 gene can be identified in other yeast species by hybridization screening. The sod2 sequence shown in the Sequence

Listing, or an oligonucleotide portion thereof, can be used as a hybridization probe. Indeed, the sod2 sequence has been found to hybridize with genomic DNA of S. octosporus indicating the presence of a sod2 homologue in this yeast species. For use in hybridization assays, the sod2-derived nucleotide sequence is labeled with a detectable label such as a radioisotope to produce a probe. The probe is incubated for hybridization with the nucleic acid to be tested under appropriate conditions of stringency.

The sod2 gene, or variants thereof, can be obtained in several different ways for use in the methods of this invention. The sod2 gene can be isolated de novo from S. pombe, as described in detail in the Exemplification below. Alternatively, the gene can be chemically synthesized by standard techniques for nucleic acid synthesis according to the nucleotide sequence given in the Sequence Listing or some variant thereof, as described above. The synthesis can be done, for example, in an automated DNA synthesizer employing the ß-cyanoethyl nucleotide phosphoramidite chemistry.

The deduced amino acid sequence of the gene product of sod2 is also given in the Sequence Listing. The protein is a putative proton antiport (sodium pump). A proton antiport uses the energy of an inwardly directed proton gradient across the cellular membrane to export sodium ions across the membrane and out of the cell. The sod2-encoded protein functions in various types of membranes. For example, the sod2-encoded product functions to transport ions in S. pombe and S. cerevisiae (yeast species removed evolutionarily by about 1.2 billion

years) and in plants (removed from yeast by far more time). In principle, a proton driven pump should work in any eukaryotic or prokaryotic cell with a primary proton gradient.

The sod2 protein or its equivalents may be useful in reverse osmosis to desalinate water or solutions. For this purpose, the protein can be incorporated in an appropriate osmotic membrane. The protein itself can be produced by recombinant DNA techniques or it can be chemically synthesized.

The salt-tolerance-conferring gene of this invention can be used to produce salt tolerant cells of different types. The cells can be eukaryotic or prokaryotic. Eukaryotic cells include yeast cells, plant cells and mammalian cells. The criteria for manifestation of the salt tolerant phenotype will vary with the cell type. In general, the introduced salt-tolerance-conferring gene must be expressed in the cell and the expression must be regulated, as appropriate, with respect to tissue and cell type and developmental stage. The use of appropriate genetic elements which regulate expression is clearly important, but expression of the heterologous gene can be affected by other factors such as positional effects and codon usage. Further, the greater the phylogenetic distance between the cell from which the gene is isolated and the recipient cell, the greater the probability is that manipulations will be required to achieve the desired level of regulated gene expression.

In general, a salt tolerant phenotype is dependent on a sufficiently high level of expression of the gene in the recipient cell. Sufficient levels of gene expression can be achieved in at least two

ways. A single copy (or low number of copies) of the gene can be introduced into a cell in conjunction with appropriate regulatory elements so that it is expressed at a sufficiently high level to provide the salt tolerant phenotype. Typically, this is achieved by placing the gene under the control of a strong promoter. Alternatively, the gene can be introduced into a cell in a sufficient number of copies (or in such a way that it will be amplified within the cell to a sufficient number of copies) to result in salt tolerance.

Cells can be transformed with the gene in any of the many ways available in the art. The particular method of transformation depends, inter alia, upon the type of recipient cell. In general, the gene is placed into an expression vector (e.g., a virus, plasmid, transposon or combination of these) coupled to genetic regulatory elements appropriate for the recipient cell type. For example, for transformation of plants the gene can be coupled to a plant promoter or other promoter functional in a plant cell. The recombinant expression vector containing the salt-tolerance-conferring gene is then inserted into the cell by any standard technique of infection or transfection.

The salt-tolerance-conferring genes of this invention can be used to develop new animal and plant varieties which exhibit increased survivability or productivity in high sodium or lithium environments. For example, genes which confer salt tolerance can be used to produce new yeast varieties for growth in culture at high salt concentrations. These new varieties can allow fermentation with water or feedstocks having significant salinity.

New plant varieties can be produced which are tolerant of high sodium concentration in soil. These new varieties will enhance crop cultivation in arid or semi-arid areas using saline waters for irrigation or allow for production of crops in areas of increasing salinity, such as the coastal plains of California. The gene can be used to produce new salt-tolerant varieties of dicotyledonous plants such as tomatoes, cucumbers, beets, potatoes, etc., and monocotyledonous plants such as grains wheat, corn, rice, etc.

Agrobacterium tumafaciens-based vectors (Ti plasmids) can be employed for transformation of dicotyledonous plants. In a preferred embodiment, the gene is inserted into the T DNA region of a binary Ti plasmid containing an appropriate selectable marker such as the gene encoding kanamycin resistance. The gene is placed under the direction of an appropriate promoter such as the cauliflower mosaic virus 35S promoter. The plasmid is inserted into Agrobacteria. The Agrobacteria carrying the recombinant Ti plasmid are used to infect plant cells (e.g., leaf cells) by co-growing the plant cells and bacteria in culture. The infected plant cells incorporate the modified T DNA into their chromosomes. Transformed cells can be selected based upon the marker gene. Standard procedures are used to regenerate the transformed plant cells from callus growths in culture to plantlets and plants. The regenerated plants produce seed capable of germinating into plants of the sa[illegible] tolerant variety.

A wide range of alternative DNA-mediated transformation techniques are available including

particle bombardment, electroporation and microinjection. For example, monocotyledonous plants that are refractory to Agrobacterium-mediated transformation can be transformed by particle bombardment. See Gordon-Kamm, W.J. et al., (1990) The Plant Cell 2:603-618.

The invention is illustrated further by the following Exemplification.

Exemplification

I. Identification and Isolation of sod2 Gene

A. Isolation of sod2-1 Sodium-Tolerant Strain of S. pombe.

The tolerance of, and ability to grow in, solutions containing high concentrations of NaCl, KCl or LiCl at various pH levels for wild type (strain 972) Schizosaccharomyces pombe was evaluated by plating actively growing cells onto Edinburgh Minimal Medium (EMM) agar plates [EMM. per litre of water: 3 g potassium hydrogen pthalate; 1.8 g disodium hydrogen phosphate (anhydrous); 5 g ammonium chloride; 20 g glucose; 20 ml salt solution (per 2 litre: 107 g $MgCl_2$ 6 H_2O, 2 g $CaCl_2$, 100 g KCl, 4 g Na_2SO_4); 1 ml vitamin solution (per 500 ml: 5 g inositol, 5 g nicotinic acid, 0.5 g calcium pantothenate, 5 mg biotin); 0.1 ml trace minerals (per 200 ml: 1 g H_3BO_3, 1.04 g $MnSO_4$ $4H_2O$, 0.8 g $ZnSO_4$ 7 H_2O, 0.4 g $FeCl_3$ 6 H_2O, 0.228 g $KMnO_4$, 80 mg $CuSO_4$ 5 H_2O, 2 g citric acid, 20 mg KI) (Mitchison, J.M., (1970) Physiological Methods for Schizosaccharomyces pombe In Methods in Cell

Physiology (ed. D.M. Prescott) pp. 131-165, Adacemic Press, London; Nurse, P.N., (1975) Nature 292:547-551) supplemented with various concentrations of the appropriate test salt. Colony growth and survival were monitored over a period of several days. Relative growth rate was estimated by measuring colony diameter with an ocular micrometer at 48 hr. Multiple colonies for measurement were chosen at random. The experiment was internally controlled for slight differences in growth rate between different plates by plating the tested strains in different regions of the same plate. Typical data are shown in Figure 1A-C. NaCl and LiCl tolerance were found to be markedly affected by pH, behaving in a parallel fashion but with LiCl being considerably more toxic than NaCl. At high sodium and lithium concentrations cellular growth rates were impaired or, at the highest levels, cells were killed. KCl tolerance was not affected markedly by pH.

Mutants capable of growing under high NaCl conditions were isolated. A typical genetic screen was as follows. Rapidly growing wild type S. pombe cells (strain 972), which are freely available from the American Type Culture Collection under accession numbers ATCC 24969, ATCC 26189 and ATCC 38366, were harvested by centrifugation and resuspended in 0.1 molar sodium acetate pH4 containing 0.4 mg/mL nitrosoguanidine for mutagenesis. Cells were allowed to sit in the dark for 30 min. and then they were washed several times with distilled water by centrifugation. Cells were then plated at densities ranging from 10^6 to 10^7 cells per plate on EMM agar

plates (pH5.5) supplemented with LiCl at 30 mM. Non-mutagenized wild type cells are killed by these conditions. LiCl was chosen because the wild type growth response to LiCl at various pH level parallels that to NaCl yet LiCl is far more toxic and thus avoids concentration-dependent osmotic effects which complicate the screen.

After incubation for several days survivors were transferred to EMM plates for further analysis. A total of 20 strains were collected. Most mutant strains were unstable and upon incubation on EMM lost their LiCl tolerance as determined by subsequent retesting. A number of relatively stable strains were screened for NaCl tolerance and strains growing on EMM plates supplemented with high levels of NaCl were isolated. In general, lithium tolerance and sodium tolerance are always found in the same strain. No lithium tolerant, sodium sensitive strains were found.

Following outcrossing and reisolation of sodium and lithium tolerant strains, ten such strains were intercrossed and assigned to a single linkage group, designated sod2. Some meiotic instability was noted for all alleles. Typically a sodium resistant strain when outcrossed to wild type would segregrate 2:2 strong sodium or lithium resistance to wild type or weak sodium or lithium tolerance. A number of cells displayed intermediate levels of resistance. This is probably explained by unequal crossing-over at an amplified locus.

A typical allele sod2-1 can grow on higher concentrations of sodium than wild type cells (Figure 2). This resistance is sodium specific and does not affect potassium tolerance.

Strain sod2-1 was crossed to ade6-210 to create a sod2-1 ade6-210 double mutant which was in turn used to complement ade6-216 in a diploid. The diploid created, sod2-1/wild type ade6-210/ade6-216, was LiCl and NaCl resistant demonstrating that the mutation was dominant.

To confirm the sodium tolerance of the strain in liquid culture, wild type and mutant and psod2-containing (psod2 is a plasmid carrying the sod2 gene, see molecular biology section below) cell lines were incubated in EMM supplemented with various concentrations of NaCl (Figures 3A-C). Each cell type was grown overnight in EMM, concentrated by centrifugation and then resuspended in a series of flasks in EMM supplemented as indicated. The flasks were then placed in a gyratory shaker at 30°C and after a 4 hr stabilization period aliquots were removed over time as indicated for cell number determinations in a Coulter Counter. The results are shown in Figures 3A-C. A. wild type; B. sod2-1; C. psod2 ura4-D18. Sod2-1 and psod2 ura4-D18 were markedly more resistant to NaCl than was the wild type. To ensure that a generalized osmotic response was not involved, strains were tested for KCl tolerance. No effect on KCl tolerance was observed. To confirm that acidification of the media did not affect these latter results the pH was monitored after termination of the experiment and found to be typical of EMM (pH 5.1-5.6) in the various cultures. Similar experiments were performed to test for the effect of the cation Cl^- versus $SO_4^=$. No major difference was found (Figures 4A-C).

Overall these data show that sod2-1 or cells with multiple copies of the psod2 plasmid are tolerant of sodium irrespective of the anion (Cl^- or $SO_4^=$) and that the response to K^+ is unaffected. Further, the rapid growth rate of sod2-1 or psod2 ura4-D18 in concentrations of Na^+ which kill wild type suggests that the energetic cost of this resistance to the cell is low.

^{22}Na Transport Studies

Sodium transport studies in wild type and mutant strains were undertaken. ^{22}Na uptake and export experiments were performed to compare sod2-1 with wild type cells. For uptake studies cells were washed and resuspended in 5 mM MES (2-(N-morpholino)-ethanesulfonic acid), 5mM PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid)) buffer at pH 5 and containing 5mM NaCl and 1µCi per ml of ^{22}Na. A kinetic analysis of uptake in wild type cells had previously shown that the uptake rate was saturated at this level of exogenous NaCl. Following incubation, aliquots of the cultures were collected by filtration then washed with a LiCl stop solution and the radioactivity in the cells quantitated in a scintillation counter. At the points indicated in Figures 5 and 6, the cultures were split and amiloride (100µM), CCCP (carboxyl cyanide m-chlorophenyl hydrozone) (6µM) or both amiloride and CCCP were added. Sampling then continued in the parallel cultures as indicated. Sodium uptake is expressed as net moles Na per cell. When wild type and sod2-1 were compared under these conditions, sod2-1 cells were found to have a far lower net

uptake rate than wild type cells (Figures 5 & 6). Since these were net uptake experiments, the difference in level between wild type and sod2-1 could have been due either to reduced uptake rates or increased export rates. Whichever the case, the experiment provides a simple explanation for the NaCl tolerance.

Sodium export rates were measured by pre-loading the cells (either wild type or sod2-1) with ^{22}Na in MES/PIPES as described above at pH 7.0 (conditions under which net sodium uptake rates are high) and ther ashing the cells by filtration and resuspending them in MES/PIPES plus 5 mM non-radioactive NaCl at pH 7.0 or pH 5.0. The ^{22}Na content of the cells was then followed by sampling aliquots of the cultures by filtration at timed intervals. Sod2-1 exported ^{22}Na at a higher rate than wild type (Figure 7).

B. Molecular Characterization of sod2 Gene

If the sod2 gene represents n export pump system or regulator of such a system then overexpressing the wild type version of the gene on a multiple copy plasmid should be sufficient to protect a cell from high Na^+ or Li^+ environments. Sod2$^+$ ura4-D18 S. pombe were therefore transformed with an S. pombe genomic DNA library in plasmid vector pFL20 (Clark, L. et al., (1986) Proc. Natl. Acad. Sci. USA 83:8253-8257). Cells were transformed using NovoZyme 234 (Novo Industries) for cell wall removal and protoplast formation (Beach, D. and Nurse, P., (1981) Nature 290:140-142). Cells were plated on media (EMM with appropriate auxotrophic supplements plus 1.2M sorbitol) lacking uracil. Surviving strains (those

carrying a plasmid complementing the uracil auxotrophy) were subsequently replica-plated to EMM plates with supplements and 30 mM LiCl plates for testing.

Two survivor yeast strains were isolated and plasmids prepared as follows. Cells were washed in 20 mM tris(hydroxymethyl)aminomethane, 50 mM ethylenediaminetetraacetic acid, pH 7.4 then resuspended in the same buffer and broken by vortex mixing with an equal volume of 400 micron glass beads. The supernatant was collected, phenol/chloroform extracted and the nucleic acids precipitated with isopropanol. The precipitate was redissolved in 10 mM tris(hydroxymethyl)aminomethane, 2 mM ethylenediaminetetraacetic acid, pH 7.4, digested with ribonuclease A then proteinase K, phenol/chloroform extracted and precipitated with ethanol. The nucleic acid pellet was redissolved as before and transformed into E. coli JM109 made competent by $CaCl_2$ washes. The E. coli were plated on L-broth containing 40µg/mL ampicillin. A bacterial colony from each preparation was then used to prepare plasmids. Two plasmids were obtained, both representing the same sequence as judged by digestion with a variety of restriction endonucleases. The yeast genomic DNA insert was 5.8 kb long. The plasmid was designated psod2 (Figure 8). The genomic insert is a Sau3A fragment inserted in the pFL20 BamHI site. All units are in kilobases. E=EcoRI; H=HindIII; B=BamHI; S=SphI.

The psod2 plasmid by itself was capable of transforming wild type S. pombe cells to lithium and sodium tolerance (Figure 3c).

Subcloning of a 2.3 kb HindIII fragment from psod2 into pFL20 showed sodium tolerance activity to be located in this portion of the psod2 plasmid. An EcoRI-SalI fragment flanking the 2.3 kb HindIII portion from the 5.8 kb-pFL20 isolate was subcloned into pUC118 and a nested set of deletions made using exonuclease III from the SalI end (Henikoff, (1984) Gene 28:351, in kit form from Promega). The HindIII 2.3 kb fragment was subcloned into pUC119 and a similar set of deletions made in the opposite orientation. Each construct was tested for sodium/lithium tolerance activity following cotransformation with pWH5 (Wright, A. et al., (1986) Plasmid 15:156-158) into a sod2+ leu1-32 strain. Cotransformation was necessary since pUC118/119 does not carry a selectable auxotrophic marker for yeast. The results for some of these deletions, defining the upstream and downstream requirement for the active gene are shown in Figure 9. Figure 9 also contains an interpretation of the gene structure based on sequence analysis. The structure of the sod2 gene as determined by sequence, S_1, primer extension and Northern Blotting analyses as well as functional testing is shown. A positive score indicates survival and growth on the LiCl plate.

Using the same sets of nested deletions in pUC118/119, the gene was sequenced using a dideoxyribonucleotide triphosphate/Sequenase sequencing protocol (Applied Biosystems). Sequence was obtained for overlapping clones in both directions for some 2400 bp starting just outside the upstream HindIII site. Upstream and downstream are relative to the deduced open reading frame. The

nucleotide sequence of the gene and deduced amino acid sequence of the encoded protein are shown in Figure 10.

The active region contains an open reading frame extending from position 188 to position 1668 bp if one allows for a short intron (position 312-388). S1 analysis using a single stranded probe synthesized from an oligonucleotide primer situated at position 795 to 812 (oligonucleotide sequence 5'ggaaccgccattccatc-3') and extending to either the upstream HindIII site (position 1) or an NcoI (position 462) site located the 3'-splice junction of the intron near position 390. The existence of the intron was confirmed by direct messenger RNA sequencing as follows. RNA was prepared from sod2-1 and passed over an oligo U Sepharose column (Pharmacia) to prepare a fraction enriched in polyadenylated messenger RNA. This RNA was used as template for reverse transcriptase primed by an oligonucleotide primer, 5'-tttagcagcatgaggccc-3' (position 415-436). The sequencing reaction was based on the reverse transcriptase DNA sequencing kit from Promega, purchased through Fisher Scientific. The sequence confirmed the position of the intron as shown in Figure 9 and 10. Downstream of the gene, deletion from position 2063 is active; a deletion from 1600 has a small amount of activity and deletion from 1436 is inactive. Upstream deletion to position 179 is active; deletion to 207 is not.

The structure of the gene was further confirmed by sequencing a cDNA clone in pTZ19R (see section III.A.1). In this clone the intron structure as described above was confirmed, the polyadenylation

site was shown to be at nucleotide 1924 and the upsteam start site at position 85. The activity of constructs deleted to position 179 therefore is presumably due to run on transcription from promoters in the vectors. Furthermore, the entire sequence of the cDNA was determined and the deduced amino acid translation product was the same as that designated sod2. Since this cDNA was prepared from a sod2-1 strain it shows that the nature of the mutation is the amplification of a wild type sequence e.g. the same as that isolated from the pFL20 gene bank.

Relationship of Plasmid to sod2-1 Mutant Strain

The 2.3 kb HindIII fragment in pWH5 was transformed into leu1-32 yeast and an integrant selected by culture on yeast extract media and then on EMM. Similarly, the 5'-3.5 kb EcoRI-HindIII fragment from psod2 was integrated. Each strain was crossed to sod2-1 and the segregation of the leu1-32 marked plasmid integrant and the lithium resistance analyzed. For the 2.3 kb HindIII fragment reassortment of the lithium resistance to all progeny made the analysis meaningless. This was presumed to be due to unequal crossing over with the amplified (see below) sod2 locus in the sod2-1 strain. For the 3.5 kb EcoRI-HindIII integrant the leu1-32 marker integrated at the same site as the 2.3 kb fragment. Based on these data and the Southern blotting results described below, but recognizing that the amplified nature of the locus in sod2-1 results in unusual recombination frequencies, it is believed that the psod2 plasmid represents the sod2 locus.

Southern blotting was used to examine the organization of the gene represented by the psod2 plasmid. Typically DNA was prepared from both wild type and sod2-1 cells, digested with a variety of restriction endonuclease enzymes, electrophoresed and transferred to GeneScreen membranes (New England Nuclear) by Southern blotting. A blot of wild type DNA and of sod2-1 DNA were hybridized (hybridization: 3 x SSC (1 x SCC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.4) 65°C overnight; final wash 0.1 x SSC, 65°C) with the nick translated 5.8 kb genomic insert from psod2. Restriction fragments hybridizing to the radioactive probe were identical in wild type DNA and in sod2-1 DNA (Figures 11A & B respectively). Lanes 1 and 14, HindIII digested lamda DNA. Remainder of lanes yeast genomic DNA: lane 2, HindIII; lane 3, HindIII/PstI; lane 4, PstI; lane 5, PstI/PvuII; lane 6, PvuII; lane 7, PvuII/HindIII; lane 8, PvuII/EcoRI; lane 9, EcoRI; lane 10, EcoRI.PstI; lane 11, EcoRI/HindIII; lane 12, A. BamHI/HindIII B. BamHI; lane 13, A. BamHI B. BamHI/HindIII.

In sod2-1 DNA, however, some of the bands were highly amplified relative to others. Upon equivalent autoradiographic exposure of the blots, band intensity of the weaker signals of the sod2-1 DNA were the same as for wild type DNA. In addition, all similar sized bands in the wild type DNA were of similar intensity. It is, therefore, deduced that the intensity profile of the weaker signals is indicative of single copy genomic DNA fragments. In the HindIII digest of wild type DNA (Figure 11A) five bands are visible: the 3.5 kb, 2.3 kb and 0.3 kb

fragments identified in the psod2 plasmid as well as bands at 1.5 kb and 9 kb which are not present in plasmid digests. These same bands are present in the sod2-1 DNA, however, the 3.5 kb, 2.3 kb and 0.3 kb are highly amplified (Figure 11B). The entire region represented by the psod2 clone appears to be amplified. There are additional non-amplified bands, however, and these appear to represent a second copy of some portion of the psod2 sequence.

When the same blots were hybridized with the psod2 2.3 kb HindIII sequence the additional non-amplified bands were not present. It is concluded that there is only one copy of the psod2 2.3 kb HindIII fragment in the S. pombe genome (Figure 11c).

Intact wild type and sod2-1 DNA was electrophoresed, Southern blotted and probed with the HindIII 2.3 kb fragment. No trace of a rapidly migrating episomic or plasmid band could be detected. It is concluded that the amplification of the sod2 sequence is chromosomally located or present on very large episomic fragments.

A gene bank was prepared in plasmid vector pWH5 using genomic DNA from sod2-1. The gene bank was used to transform wild type cells to lithium/sodium resistance at high frequency consistent with the amplified nature of the sod2 locus in sod2-1.

Construction of a Gene Disruption, Inactivated sod2 Mutation

The HindIII 2.3 kb fragment of psod2 was subcloned into pUC118 and transformed into dam$^-$ JM103 E. coli. The plasmid was isolated and digested with

restriction endoclease BclI to remove a fragment extending from position 1155 within the open reading frame to position 1955 downstream of the open reading frame. Approximately one half of the open reading frame was thus deleted. The ura4 gene from S. pombe was isolated as a HindIII fragment from plasmid pura4. HindIII/BamHI oligonucleotide linkers were ligated to the isolated ura4 gene and the ura4 gene then ligated into the BclI sites of the HindIII 2.3 kb fragment in pUC118. Following preparation, the resulting plasmid was transformed into an S. pombe diploid strain of genetic constitution ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-210/ade6-216 and plated onto EMM plus 1.2 M sorbitol plus 100 g/mL leucine plates. Following selection for integration 120 strains displaying prototrophy for uracil were characterized for sodium and lithium tolerance.

Seven strains were isolated which grew poorly on EMM and could not grow on EMM Plus 150 mM NaCl. These strains were presumed to represent gene replacements resulting from double homologous recombination events or insertions at sod2 which hindered the function of the sod2 gene. These strains germinated poorly from the spore on EMM (plus leucine) however grew better on EMM (plus leucine) made by eliminating Na_2HPO_4 and replacing it with K_2HPO_4 and adjusting the pH to 5.5 Such a formulation has a final sodium concentration of approximately 0.5 mM as compared to EMM with 26 mM Na^+. The gene knockout strain sod2::ura4-D18 ura4-D18 leu1-32 ade6-210 was used to prepare DNA for a genomic Southern blot to verify the gene elimination. Genomic DNA from several strains was

prepared and digested with various restriction enzymes and a Southern blot prepared on GeneScreen following agarose gel electrophoresis. The membranes were then hybridized to the nick translated 2.3 kb genomic fragment from psod2 at high stringency. The result showed that the HindIII 2.3 kb fragment is missing from the sod2::ura4 strain (Figure 12). Lanes 1-5, BclI digested; lanes 6-10, HindIII digested. Lanes 1, 6 sod2-1; lanes 2, 7 inactivated strain K35-1; lanes 3, 8 inactivated strain K88-1; lanes 4, 9 inactivated strains K16-2; lanes 5, 10 inactivated strain K15-5. This fragment was thus disrupted. K15-5 was used for further experiments.

The phenotype of the disruptant was sodium sensitivity, lithium sensitivity and ammonium sensitivity. The latter presumably results from cytoplasmic alkalinization by ammonia and the sod2 gene, encoding a putative proton antiport, in part, plays a role in pH regulation.

Construction of an Overexpression Plasmid

Since gene amplification plays a role in sod2-1 mutant function, construction of a plasmid containing a strong promoter linked to the open reading frame from psod2 should function to cause sodium tolerance after reintegration into the genome. The plasmid pART5, a derivative of pART1 (McLeod, M. et al., (1987) EMBO J. 6:3665-3671) containing the alcohol dehydrogenase promoter was used. An NdeI site was inserted by oligomutagenesis at the putative start codon of the sod2 open reading frame at position 188 (using a mutagenic oligonucleotide 5'-ttgcctaattcatatgggctgg-3'). Subsequent to the

removal of an internal NdeI site at position 630 (using a mutagenic oligonucleotide 5'-ctggatttgcgtatgcattgt-3') the gene was excised from pUC119 as an NdeI-EcoRI fragment and ligated into pART5 to generate plasmid psod2-ADH (Figure 13).

Following construction, the plasmid psod2-ADH was transfected into a leu1-32ura4-D18ade6-210 Schizosaccharomyces pombe strain and plasmid integrated strains selected by growth on YEA medium and selection on EMM supplemented with uracil and adenine. The plasmid containing strains were then tested for LiCl and NaCl tolerance. Wild type, sod2-1 and psod2-ADH int leu1-32 strains were plated onto EMM agar plates containing LiCl at the concentrations indicated. (Figure 14: ADH1=psod2-ADH1 and ADH2=psod2-ADH2). Growth was determined as for Figure 1 and expressed relative to ADH1 on 20 mM LiCl as 100 percent. A strain overexpressing the sod2 gene behind an ADH promoter is strongly Li^+ and Na^+ tolerant. Differences in resistance among the different ADH strains presumably reflects the particular context of genomic integration in each case. Similar constructions based on inserting NdeI restriction sites at the in frame ATG at positions 670 or 830 were inactive with respect to lithium tolerance upon subsequent transformation into yeast.

A version of psod2-ADH from which the intron, as defined in Figure 10, had been removed by oligomutagenesis is also functional and confers the same level of sodium/lithium tolerance as the intron plus version of the gene.

Sodium Efflux from sod2-1 and psod-2-ADH1 Strains

Wild type, sod2-1, psod2-ADH1 and sod2::ura4 strains were labelled with ^{22}Na as described above and the export of Na from the cell monitored following resuspension in MES/PIPES buffer with 6mM NaCl or in sodium free MES/PIPES buffer. The sod2-1 and the psod2-ADH1 strains exported sodium more rapidly than wild type under both conditions. The sod2::ura4 strain exported more slowly than wild type (Figures 15, 16).

Heterologous DNA with Sequence Similarity to sod2

A Southern blot analysis of other species of yeast including Schizosaccharomyces (japonicus and octosporus) and Saccharomyces cerevisiae were performed. No hybridization signal was found for S. cerevisiae. In the case of Schizosaccharomyces octosporus a hybridization signal was found. It is concluded that these two yeasts may harbour a gene similar to sod2 from S. pombe.

II. Activity of sod2 Gene in Saccharomyces Cerevisiae

In order to show the utility of the sod2 gene in other organisms, the yeast Saccharomyces cerevisiae (evolutionarily removed by 1.2 billion years from S. pombe) was chosen for transformation.

The sod2 gene was excised from pRM1 (see section III.A.1 below) with EcoRI, blunt-ended with Klenow, linkered with BamHI linkers and inserted in both orientations in the BamHI site in the S. cerevisiae cloning vector pCGS110. In the correct orientation (plasmid pCGS110-sod2) the sod2 gene was under the control of the galactose inducible promoter Gall (Figure 18).

Both the forward and reverse orientation plasmids were transformed into the S. cerevisiae strain BF305-15d (mat a, leu2-3, leu2-112, his3-11, his3-15, trp1, ura3, arg5,6, ade1, met14, gal+). The cells were then grown on media based on yeast nitrogen base (Difco) supplemented as required and with glucose or galactose as the carbon source.

With glucose as the carbon source, sodium and lithium tolerance in S. cerevisiae is very high to begin with. Against this background no additional enhancement by sod2 could be detected even at low glucose concentrations in the presence of galactose sufficient to induce the gal1 promoter. In order to show an effect, presumably the equivalent salt tolerance mechanisms would have to be deleted from the S. cerevisiae.

With galactose as the carbon source, however, S. cerevisiae is much more sensitive to lithium. In the presence of galactose, which induces the gal1 promoter, S. cerevisiae cells carrying the pCGS110-sod2 construct (Figure 19 and Figure 20, strains 3 and 4) survive lithium concentrations which prevent colony formation for strains carrying only empty plasmid (Figure 20, strains 1 and 2) or plasmid with the sod2 gene in the reverse orientation with respect to the gal1 promoter. The rescue occurs after a considerable growth delay.

We have not investigated the physiology of these cells in detail; sod2, however, clearly rescues these cells from lithium toxicity.

Galactose grown cells are not noticeably more sodium sensitive and therefore this aspect of the ion tolerance could not be investigated. We assume that

during galactose growth some particular (possibly inducible) essential pathway is particularly lithium sensitive. Based on the physiology done in S. pombe the mechanism of resistance is likely to be at the level of maintaining low internal lithium concentrations.

We conclude that the sod2 gene can be made to function transgenically to provide ion tolerance in S. cerevisiae. The failure to enhance sodium tolerance may relate to unknown levels of expression or to failure to obtain optimum membrane insertion coupled with a high intrinsic level of sodium tolerance. We presume that pEGS110-sod2 would restore sodium tolerance to a sodium hypersensitive mutant.

III. Introduction of the sod2 Gene Into Tobacco Plants.

A. Isolation and Characterization of a sod2 cDNA Clone.

1. Construction of S. pombe mRNA-derived cDNA Library.

Total RNA was extracted from S. pombe sod2-1 mutant cells that were harvested in the logarhythmic phase of growth. The polyA(+) fraction of the RNA was isolated and used to synthesize double stranded cDNA. The cDNA was treated with EcoRI methylase and ligated to EcoRI linkers. After digestion with EcoRI to produce cohesive ends, the cDNA was ligated into the EcoRI site of pTZ19R DNA. E. coli strain DH5α was transformed by electroporation with the ligation products and cells were spread onto plates containing ampicillin for selection of transformants.

2. Screening of the Library with sod2 Genomic Clone.

The above library was screened by hybridization with radiolabelled sod2 genomic clone. Hybridization-positive colonies were rare, occurring at a frequency of less than one in 40,000 recombinants.

3. Sequencing of a cDNA Clone and Comparison with Genomic Clone.

A full length sod2 cDNA clone (pRM1) was sequenced using the chain termination method and the sequence was compared to that of the sod2 genomic clone. This comparison confirmed the existence and position of an intron spanning 75 base pairs in the genomic clone.

B. Introduction of the Coding Region of the sod2 Gene into Plant Expression Vectors.

1. The plant expression vectors described below are all based on the binary Ti plasmid-derived vector, pBIN19. (Bevan, M.W. (1984) Nucleic Acids Res. 12:8711-8721). Agrobacterium tumafaciens-mediated transformation with this vector confers kanamycin resistance to plants.

2. Site Directed Mutagenesis to Introduce Restriction Sites into sod2 DNA.

a. sod2 cDNA clone.

The NcoI site present within the coding region of the sod2 cDNA sequence was removed by oligonucleotide directed mutagenesis. The amino acid sequence was not altered by this change. An NcoI site was created at the position of the initiation

codon and a BamHI site was introduced after the termination codon. The resulting construct is called RM2.3.

b. Genomic sod2 clone including intron.

The removal and introduction of NcoI sites was performed in the same way as for the cDNA clone. A BamHI site was not introduced after the termination codon. The resulting construct is called RD1.2.

c. Genomic sod2 clone, intron removed.

The intron defined by comparison of the cDNA and genomic DNA sequences was removed from the sod2 genomic clone using oligonucleotide directed mutagenesis. The amino acid sequence was not altered. The same changes that were made to the cDNA sequence were then made to the intron-less version of the sod2 genomic clone. The resulting construct is called R4Mut3.

3. Promoters and Translational Enhancers.

Plasmid constructs were made which linked the sod2 gene to promoters of differing strengths.

a. Cauliflower mosaic virus 35S promoter (Jefferson, R.A. et al. (1985) EMBO J. 6:3901-3907).

The constructs, RM2.3, RD1.2 and R4Mut3 were cut with NcoI and BamHI (RM2.3 and R4Mut3) or with NcoI and HindIII (RD1.2). The cut DNA was ligated to NcoI-BamHI or HindIII-BamHI adapters as appropriate cut with BamHI and ligated with pBI162 DNA (available from Plant Biotechnology Institute, Saskatoon, Canada) that had been cut with BamHI. This produced three constructs for use in plant transformation: p35-RM2.3, p35-RD1.2 and p35-R4Mut3. In each of these constructs, a sod2 coding region is under the

control of the Cauliflower mosaic virus 35S promoter and a downstream transcription termination sequence from the nopaline synthase gene.

b. Tandem CaMV 35S promoter (Kay, R. et al. (1987) Science 236:1299-1302) with alfalfa mosaic virus RNA4 translational enhancer.

The constructs, RM2.3 and R4Mut3 were cut with NcoI and BamHI and ligated to pBI524 DNA (available from Plant Biotechnology Institute, Saskatoon, Canada) that had been cut with the same two enzymes. The construct pRD1.2 was cut with NcoI and HindIII, ligated to a HindIII-BamHI adapter, cut with BamHI and ligated to pBI524 DNA that had been cut with NcoI and BamHI. This produced three more constructs to be used in plant transformation: pT35AMV-RM2.3, pT35AMV-RD1.2 and pT35-R4Mut3. In each of these constructs, a sod2 coding region is under the control of a tandem version of the CaMV 35S promoter and a downstream transcription termination sequence from the nos gene. In addition DNA sequence encoding a 5' transcribed, non-translated leader sequence that confers higher levels of translation is present between the tandem 35S promoter and the NcoI site.

C. Production of Transformed Plants.

1. Introduction of p35-RM2.3, p35-RD1.2, p35-R4Mut3, pT35AMV-RM2.3, pT35AMV-RD1.2 and pT35AMV-R4Mut3 into Agrobacterium tumafaciens strain MP90 was done by a tri-parental mating with E. coli DH5α and E. coli RK2013.

2. Infection of Plant Tissue with A. tumafaciens.

Axenic Nicotiana tabacum var xanthi leaf explants were infected using standard techniques with A. tumafaciens strain MP90 bearing the above plasmids.

3. Selection of Kanamycin Resistant Transformants.

Infected tissue was cultured on MS medium (Murashige, T. and Skoog, F. (1962) Plant Physiol. 15:473-497) containing MS salts and MS vitamins, 3% sucrose, 2.5 mg/l benzyladenine, 0.1 mg/l naphthaleneacetic acid, 500 mg/l carbenicillin, pH5.6 and containing 100 mg/l kanamycin, transferred monthly to fresh medium. As shoots emerged they were removed and placed on the above medium lacking benzyladenine and with half the amount of both carbenicillin and kanamycin. If roots formed, plants were transferred to soil.

4. Re-testing Regenerants for Kanamycin Resistance.

When plants were transferred to soil, leaf explants were placed on the original selection medium. Plants which produced callus in the presence of kanamycin were considered to be confirmed as transformants.

D. Initial Assessment of Transformed Tobacco Plants for Expression of the sod2 Gene.

1. Presence of sod2 Transcript in Transformed Plants.

Total RNA was isolated from leaves of transformed and control plants. The RNA was treated with DNAase to remove any contaminating DNA. First strand cDNA was synthesized by reverse transcriptase

from total RNA primed with oligo(dT). Sod2 mRNA-derived DNA was amplified by the polymerase chain reaction (PCR) using sod2 specific primers. This reaction was carried out in the presence of [α^{32}P]dCTP and the presence of the PCR product monitored by autoradiography. Negative controls included RNA extracted from non-transformed plants and reactions in which reverse transcriptase was omitted from the cDNA synthetic reaction. The presence of a PCR product of the correct size synthesized from cDNA derived from transformed plants and the absence of the PCR product in the control reactions confirms the presence of sod2 transcript in transformed plant tissue.

2. Production of Callus and Regeneration of Shoots From Transgenic Tissue in the Presence of LiCl.

At the time that plants were re-tested for kanamycin resistance they were also placed on the original selection medium, lacking kanamycin and including various levels of LiCl. We have observed callus formation and regeneration of plants from transformed tissue placed on levels of LiCl as high as 130 mM. We have observed no regeneration of control, non-transformed tissue in the presence of this level of LiCl. These observations are consistent with the phenotype that we expect for plants expressing the yeast sod2 gene.

IV. Introduction of the sod2 Gene into A. thaliana Plants.

A. Production of Transformed Plants. (Modification of Renate Schmidt and Lothar Willmitzer (1988) Plant Cell Reports 7:583-586).

1. Infection of Plant Tissue with A. tumafaciens. Arabidopsis thaliana var RLD.

Leaf explants were pre-cultured for two days on MS salts plus B5 vitamins, 3% sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid, 0.2 mg/l kinetin, 0.8% agar, pH5.6. Leaf explants were then co-cultured for a further two days with A. tumafaciens bearing the above plasmids and a tobacco cell feeder layer.

2. Selection of Kanamycin Resistant Transformants.

Explants were transferred to selection medium containing MS salts, B5 vitamins, 3% sucrose, lmg/ml benzyladenine, 0.1 mg/l naphthaleneacetic acid, 500 mg/l carbenicillin, 25 mg/l kanamycin, 0.8% agar, pH5.6. When shoots are produced, they were placed in shoot elongation medium containing MS salts, B5 vitamins, 3% sucrose, 1 mg/l benzyladenine, 0.4 mg/l naphthaleneacetic acid, 0.1 mg/l Gibberellic acid, 500 mg/l carbenicillin and 0.8% agar, pH5.6. When shoots were 3 to 4 cm long they were transferred to rooting medium containing 0.5X MS salts, B5 vitamins, 1 mg/l indole-3-butyric acid, 250 mg/l carbenicillin, 0.8% agar, pH5.6. Plantlets were grown axenically until mature and seeds (R2) were collected from individual plants.

3. Production of R_3 Seeds.

R2 seeds were germinated on medium containing MS salts, B5 vitamins, 25 mg/l kanamycin, 0.8% agar, pH5.6. Seedlings which developed beyond the four leaf stage were planted in soil and allowed to mature. Seeds (R3) from individual plants were collected.

4. Re-testing of Putative Transformants for Kanamycin Resistance.

R3 seeds from each independently transformed line were placed on germination medium containing MS salts, B5 vitamins, 25 mg/l kanamycin, 0.8% agar, pH5.6. Seeds which germinated and developed past the dicot stage were deemed to be kanamycin resistant. R3 seed lines that were true breeding for resistance to kanamycin were selected for further analysis.

B. Initial Assessment of Transformed A. thaliana Plants for Expression of the sod2 Gene.

1. Testing for Presence of sod2 Transcript in Transformed Plants.

The same procedure that was used to identify sod2 transcript in tobacco plants was used for A. thaliana plants. Transcript was present in tissue from transformed plants. The level of transcript present varied from plant to plant.

2. Testing Germination and Growth in Presence of LiCl.

R3 seeds were placed on germination medium lacking kanamycin and supplemented with 12.5 mM LiCl. Under these conditions one of the transgenic

lines (#75) tolerated the LiCl better than control plants and at the four leaf stage had produced plants with larger and greener leaves than controls. These plants were transferred to soil for seed production at the six leaf stage. The line which showed the most promising growth in the presence of LiCl also had the highest level of sod2 transcript present. This observation is consistent with the expected effect of increasing the level of expression of the sod2 gene in the transformed plants.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

Sequence Listing

Sequence ID No.: 1

Sequence Type: Nucleotide with corresponding protein

Sequence Length: 2331

Molecule Type: genomic DNA

Original Source Organism: yeast S. pombe

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aagctttgtt actccaatca aaaagataac taaggtaccc cggtcctcaa gtataaacca      60

caggcatgca cgcatcagtc cgtggctaac tgtatctttt gccacatttt atgtcgaata     120

ctctaaaaaa aatattatag gaatttatta caagaaaaca ttctcttgtg gatattgcct     180

aattact ATG GGC TGG AGA CAA CTT GAT ATA GAC AAA GTC CAT TTA GCT       229
        Met Gly Trp Arg Gln Leu Asp Ile Asp Lys Val His Leu Ala

TTA ATA GTG GCC GGG GGA TTT ATA ACA TTT TTC TGC TAT TTT TCA GAA       277
Leu Ile Val Ala Gly Gly Phe Ile Thr Phe Phe Cys Tyr Phe Ser Glu

GTT TTT CGA AAA AAA TTA CTA GTT GGA GAA GCT G gtacgttgaa              321
Val Phe Arg Lys Lys Leu Leu Val Gly Glu Ala

gatattgtat agggtggttt ttgaaaatta gcaattgata taaaataaga ctaatactag     381

tgtgtag TT CTT GGA AGT ATC ACT GGA TTA ATA TTT GGG CCT CAT GCT        429
       Val Leu Gly Ser Ile Thr Gly Leu Ile Phe Gly Pro His Ala

GCT AAA CTC GTA GAC CCT TTT TCC TGG GGT GAC CAT GGA GAT TAC TTG       477
Ala Lys Leu Val Asp Pro Phe Ser Trp Gly Asp His Gly Asp Tyr Leu

ACA GTA GAG ATT TGT AGA ATC GTA CTT GAT GTG CGT GTG TTT GCT TCT       525
Thr Val Glu Ile Cys Arg Ile Val Leu Asp Val Arg Val Phe Ala Ser

GCA ATA GAA CTC CCC GGT GCA TAT TTT CAA CAT AAT TTT CGA AGC ATC       573
Ala Ile Glu Leu Pro Gly Ala Tyr Phe Gln His Asn Phe Arg Ser Ile

ATT GTA ATG CTA TTA CCA GTT ATG GCT TAC GGG TGG TTA GTT ACA GCT       621
Ile Val Met Leu Leu Pro Val Met Ala Tyr Gly Trp Leu Val Thr Ala

GGA TTT GCA TAT GCA TTG TTT CCA CAA ATT AAC TTT TTA GGA TCT TTG       669
Gly Phe Ala Tyr Ala Leu Phe Pro Gln Ile Asn Phe Leu Gly Ser Leu

CTG ATC GCA GGA TGT ATA ACT TCT ACT GAT CCT GTT CTA TCA GCA TTG       717
Leu Ile Ala Gly Cys Ile Thr Ser Thr Asp Pro Val Leu Ser Ala Leu
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ATT GTA GGA GAA GGT CCA TTA GCT AAA AAG ACT CCT GAA CGG ATT CGG      765
Ile Val Gly Glu Gly Pro Leu Ala Lys Lys Thr Pro Glu Arg Ile Arg

TCT TTA TTG ATC GCT GAG TCT GGA TGT AAT GAT GGA ATG GCG GTT CCT      813
Ser Leu Leu Ile Ala Glu Ser Gly Cys Asn Asp Gly Met Ala Val Pro

TTT TTC TAT TTT GCT ATC AAA CTT CTT ACT GTT AAG CCA TCG AGG AAT      861
Phe Phe Tyr Phe Ala Ile Lys Leu Leu Thr Val Lys Pro Ser Arg Asn

GCA GGG AGG GAT TGG GTG CTG CTT GTT GTG TTG TAT GAA TGT GCA TTT      909
Ala Gly Arg Asp Trp Val Leu Leu Val Val Leu Tyr Glu Cys Ala Phe

GGT ATA TTT TTT GGG TGT GTA ATA GGG TAT CTT TTA TCG TTC ATT TTA      957
Gly Ile Phe Phe Gly Cys Val Ile Gly Tyr Leu Leu Ser Phe Ile Leu

AAG CAC GCT CAG AAA TAC CGT TTA ATT GAT GCT ATT AGT TAT TAT TCC     1005
Lys His Ala Gln Lys Tyr Arg Leu Ile Asp Ala Ile Ser Tyr Tyr Ser

CTT CCG CTA GCG ATA CCT TTA TTA TGT TCT GGG ATA GGA ACT ATT ATT     1053
Leu Pro Leu Ala Ile Pro Leu Leu Cys Ser Gly Ile Gly Thr Ile Ile

GGA GTT GAT GAC CTG TTG ATG TCC TTT TTT GCT GGA ATA TTA TTT AAC     1101
Gly Val Asp Asp Leu Leu Met Ser Phe Phe Ala Gly Ile Leu Phe Asn

TGG AAT GAT TTA TTT TCC AAA AAT ATA TCT GCT TGT TCT GTA CCT GCT     1149
Trp Asn Asp Leu Phe Ser Lys Asn Ile Ser Ala Cys Ser Val Pro Ala

TTT ATT GAT CAG ACT TTT AGT TTA CTA TTT TTT ACC TAT TAT GGT ACA     1197
Phe Ile Asp Gln Thr Phe Ser Leu Leu Phe Phe Thr Tyr Tyr Gly Thr

ATC ATT CCC TGG AAT AAT TTT AAT TGG TCT GTT GAA GGC TTG CCT GTT     1245
Ile Ile Pro Trp Asn Asn Phe Asn Trp Ser Val Glu Gly Leu Pro Val

TGG CGT TTA ATT GTC TTT AGC ATA TTG ACT CTA GTT TGT CGT CGA TTA     1293
Trp Arg Leu Ile Val Phe Ser Ile Leu Thr Leu Val Cys Arg Arg Leu

CCG GTT GTA TTT TCG GTG AAG CCT TTA GTT CCG GAC ATT AAG ACA TGG     1341
Pro Val Val Phe Ser Val Lys Pro Leu Val Pro Asp Ile Lys Thr Trp

AAA GAA GCC CTT TTC GTT GGA CAT TTC GGA CCA ATA GGG GTT TGC GCA     1389
Lys Glu Ala Leu Phe Val Gly His Phe Gly Pro Ile Gly Val Cys Ala

GTT TAT ATG GCA TTT CTT GCA AAA TTA CTG TTG TCC CCG GAT GAA ATT     1437
Val Tyr Met Ala Phe Leu Ala Lys Leu Leu Leu Ser Pro Asp Glu Ile
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GAA AAG AGT ATT TAT GAA TCA ACT ACA GTA TTT TCA ACA CTA AAT GAA      1485
Glu Lys Ser Ile Tyr Glu Ser Thr Thr Val Phe Ser Thr Leu Asn Glu

ATA ATT TGG CCG ATC ATT TCG TTT GTT ATC TTA TCC TCA ATC ATT GTT      1533
Ile Ile Trp Pro Ile Ile Ser Phe Val Ile Leu Ser Ser Ile Ile Val

CAT GGT TTC AGT ATC CAT GTA TTA GTG ATT TGG GGA AAG TTA AAA AGT      1581
His Gly Phe Ser Ile His Val Leu Val Ile Trp Gly Lys Leu Lys Ser

CTG TAT TTA AAT CGA AAA GTC ACC AAG TCC GAT TCC GAT TTG GAG TTA      1629
Leu Tyr Leu Asn Arg Lys Val Thr Lys Ser Asp Ser Asp Leu Glu Leu

CAA GTA ATA GGG GTT GAT AAG TCA CAG GAA GAT TAC GTT taggaaagct      1678
Gln Val Ile Gly Val Asp Lys Ser Gln Glu Asp Tyr Val

cttttaatgt caattcggat ttccaaatta ttttcaaatg tattgtgaat cgctgtctct    1738

ggtcaaaaag attactgcac tcatattttg aaattccttc tatagttgat atatactata    1798

agataagtga ttctcagaat cacaaggcta accaccaaca gggatggagt gtatattttt    1858

gttgtacata tatattatct acaatagagt aattttcggc ttctataatt catttatttt    1918

cttactactc taaaaatatt gtataatttc taaaactgat caagatactg agaaaagtac    1978

aaatcgttat ttaatttgta atttattttg atggctaaaa cttaccaata ttcgttcgct    2038

tcaaacaata ccaatcttac gaaacacctt acgcttcatc aaagtctact ttggatcacc    2098

taatatttta ttatttgttg ttgtaattat acaaactaat actattttat gtaagaaact    2158

aagaaaacgg aaaatcaata gctactttgt gtatataaat agcaatcaaa ttaaaacttg    2218

ataaatctca cctactaaaa cacatcaacg tacttcaaag ggcctaacta ctataagact    2278

tggtaatatt taaatagtgt ttctattagt aggtagcttc aaagtatgta taa...aagctt  2331
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CLAIMS

1. An isolated gene, or fragment thereof, which confers sodium or lithium tolerance upon a cell.

2. A gene of claim 1, having the sod2 nucleotide sequence shown in Sequence Listing 1.

3. A gene of claim 1, wherein the cell is the fission yeast cell, S. pombe.

4. A gene of claim 1, wherein the cell is a yeast, S. cerevisiae.

5. A gene of claim 1, wherein the cell is a plant cell.

6. An isolated gene having the sod2 nucleotide sequence shown the Sequence Listing.

7. Isolated protein encoded by a gene which confers sodium tolerance upon a cell.

8. Isolated protein of claim 7, having the amino acid sequence shown in the Sequence Listing.

9. An expression vector containing a gene, or fragment thereof, which confers sodium tolerance upon a cell.

10. An expression vector of claim 10, wherein the gene is under the control of a strong promoter.

11. An expression vector of claim 11, wherein the promoter is a yeast promoter.

12. An expression vector of claim 11, wherein the promoter is a plant promoter.

13. A plasmd having alcohol dehydrogenase promoter fused to the sod2 nucelotide sequence shown in the Sequence Listing.

14. A plasmid, psod2, consisting of a 5.8 kb S. pombe wild type genomic DNA insert in plasmid vector pFL20, which is capable of conferring sodium and lithium resistance upon wild type S. pombe and S. cerevisiae.

15. A recombinant Ti plasmid containing, in its T region, a gene conferring sodium tolerance under the control of a promoter functional in a plant cell and a selectable genetic marker.

16. A recombinant Ti plasmid of claim 15, wherein the gene conferring sodium tolerance has the sod2 nucleotide coding sequence shown in Sequence Listing 1.

17. A cell transformed with the gene of claim 1.

18. A cell of claim 17, wherein the gene has the nucleotide coding sequence given in Sequence Listing 1.

19. A cell of claim 17, which is a plant cell.

20. A cell of claim 17, which is a yeast cell.

21. A sodium tolerant strain of S. pombe, sod2-1, ATCC _____.

22. A culture of yeast cells transformed with a gene which confers sodium or lithium tolerance.

23. A plant transformed with a gene which confers sodium or lithium tolerance.

24. A plant of claim 23, wherein the gene has the sod2 nucleotide sequence shown in the Sequence Listing.

25. A seed transformed with a gene which confers sodium tolerance, the seed being capable of germinating into a sodium tolerant plant.

26. A seed of claim 25, wherein the gene has the sod2 coding nucleotide sequence shown in the Sequence Listing.

27. A method of conferring tolerance to sodium or lithium upon a cell, comprising transforming the cell with a gene that confers sodium or lithium tolerance.

28. A method of claim 27, wherein the gene is sod2 or a variant thereof.

29. A method of claim 28, wherein the gene has the nucleotide sequence shown in the Sequence Listing.

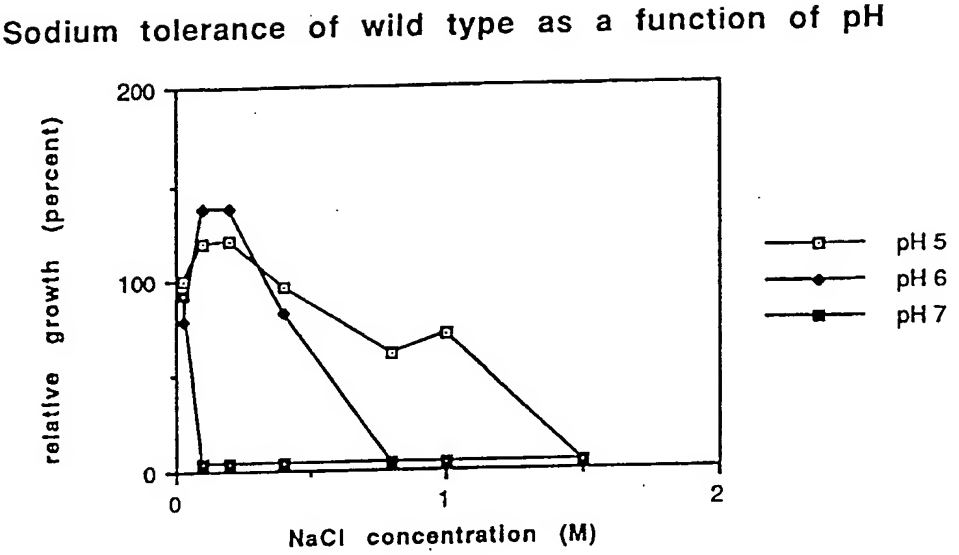

Fig. 1A

Lithium tolerance of wild type as a function of pH

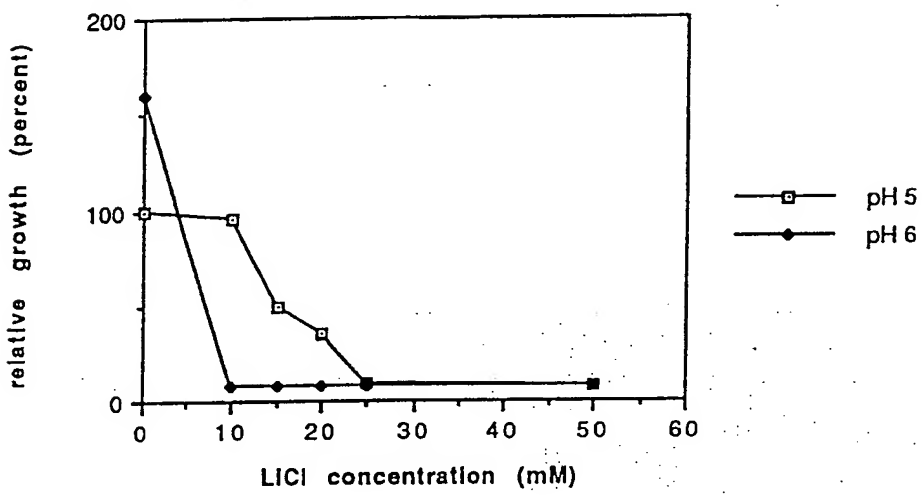

Fig. 1B

Potassium tolerance of wild type as a function of pH

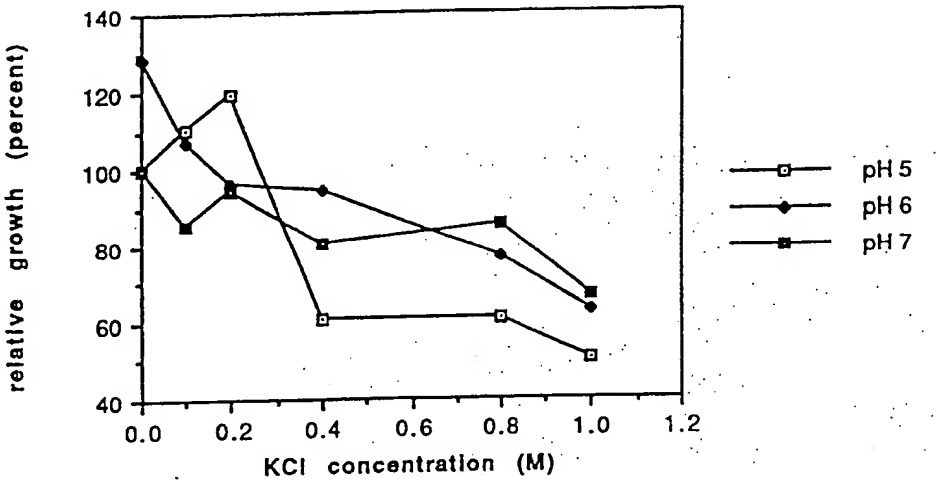

Fig. 1C

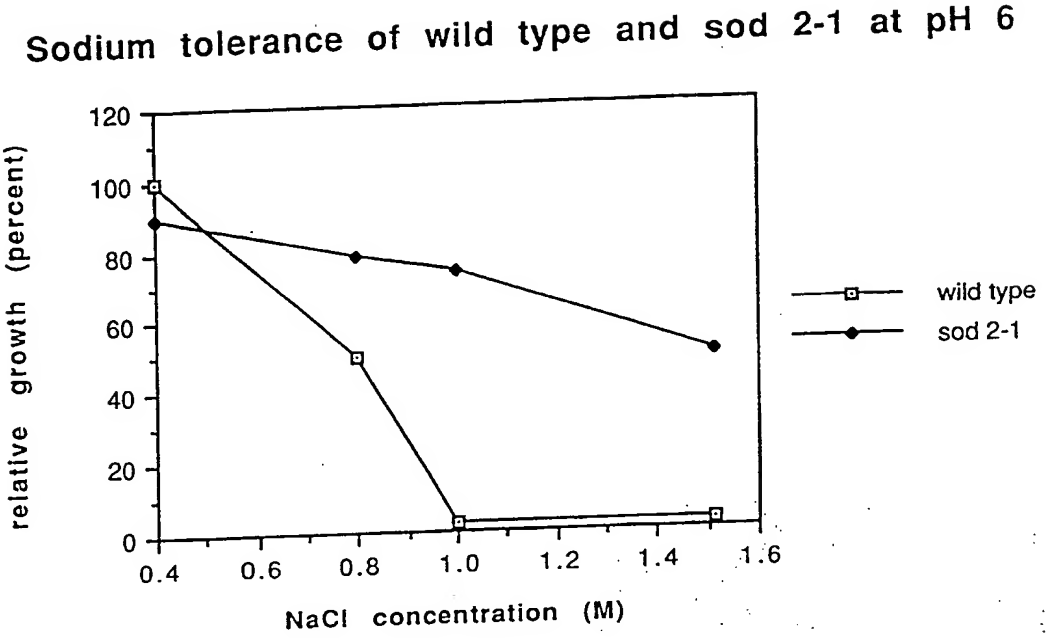
Sodium tolerance of wild type and sod 2-1 at pH 6
relative growth (percent)
120
100
80
60
40
20
0
0.4
0.6
0.8
1.0
1.2
1.4
1.6
NaCl concentration (M)
wild type
sod 2-1

Fig. 2

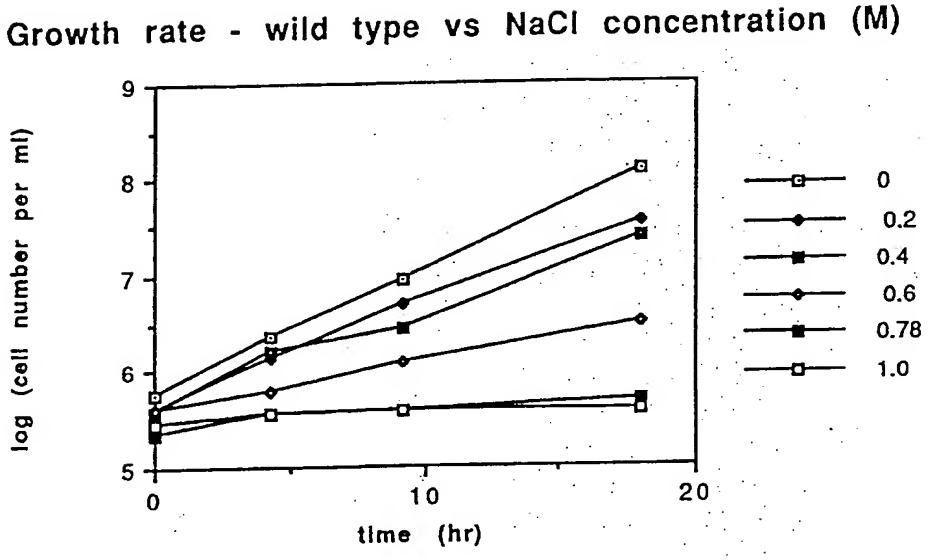
Growth rate - wild type vs NaCl concentration (M)
log (cell number per ml)
9
8
7
6
5
0
10
20
time (hr)
0
0.2
0.4
0.6
0.78
1.0

Fig. 3A

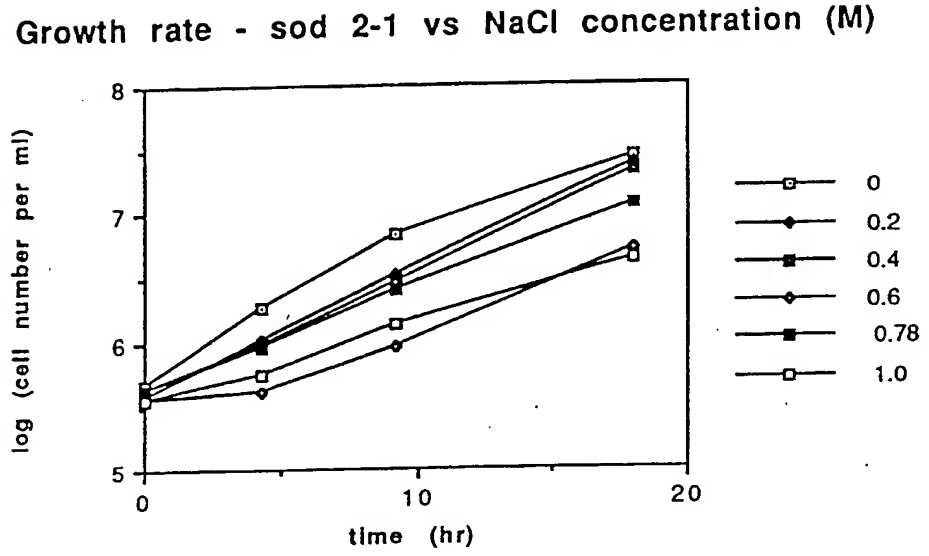
Growth rate - sod 2-1 vs NaCl concentration (M)
log (cell number per ml)
8
7
6
5
0
10
20
time (hr)
0
0.2
0.4
0.6
0.78
1.0

Fig. 3B

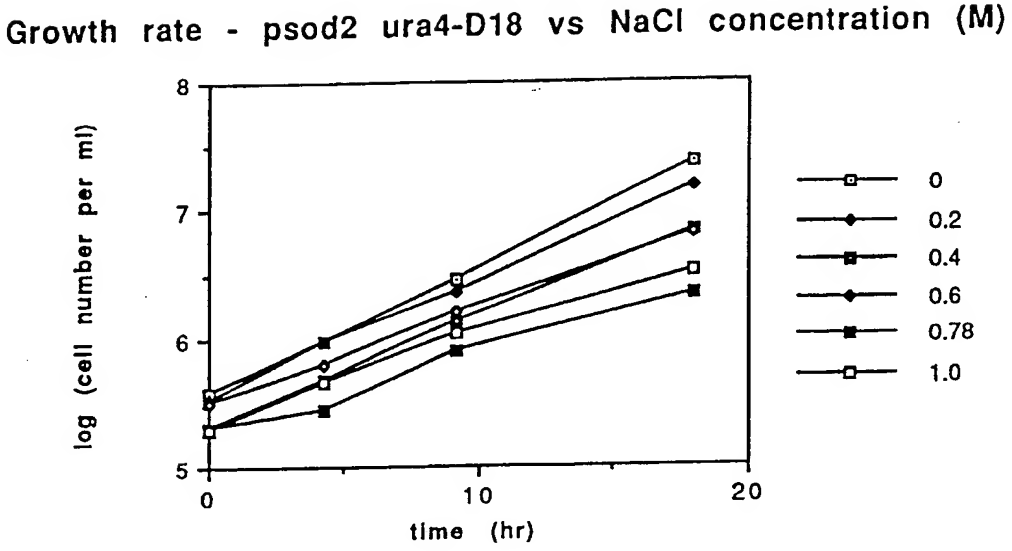
Growth rate - psod2 ura4-D18 vs NaCl concentration (M)
log (cell number per ml)
8
7
6
5
0
10
20
time (hr)
0
0.2
0.4
0.6
0.78
1.0

Fig. 3C

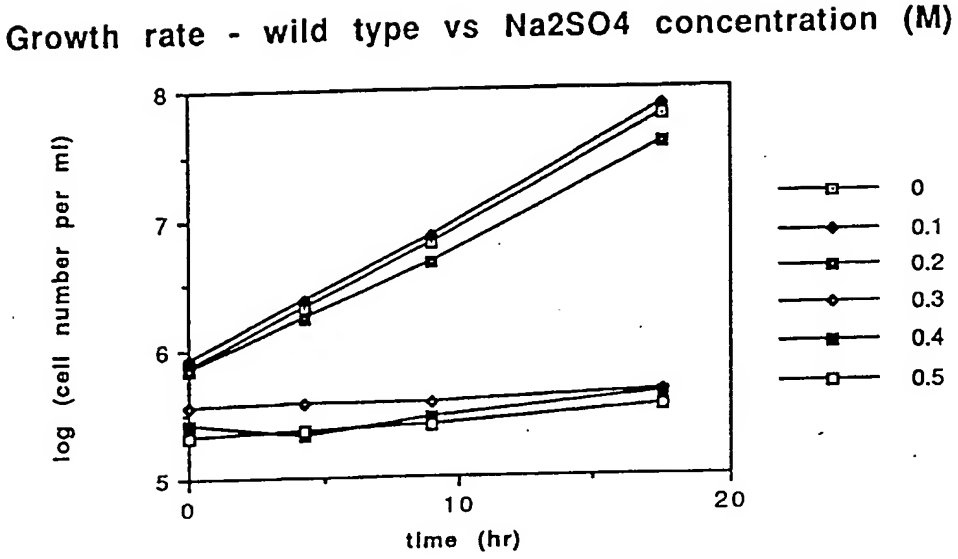
Growth rate - wild type vs Na2SO4 concentration (M)
log (cell number per ml)
8
7
6
5
0
10
20
time (hr)
0
0.1
0.2
0.3
0.4
0.5

Fig. 4A

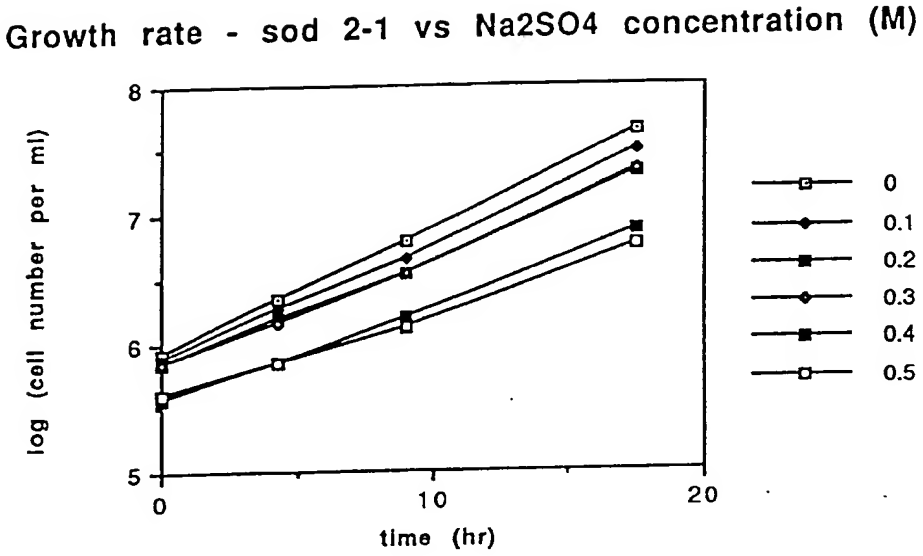
Growth rate - sod 2-1 vs Na2SO4 concentration (M)
log (cell number per ml)
8
7
6
5
0
10
20
time (hr)
0
0.1
0.2
0.3
0.4
0.5

Fig. 4B

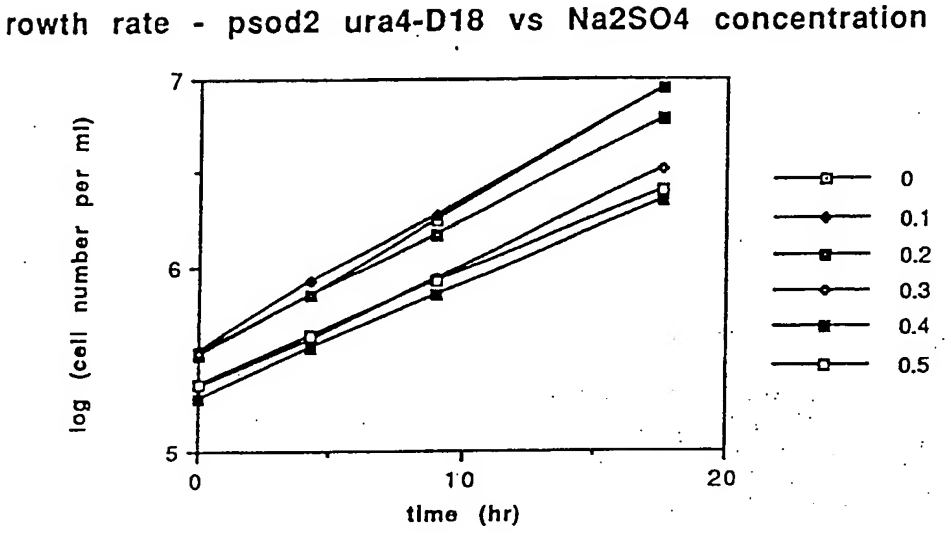
rowth rate - psod2 ura4-D18 vs Na2SO4 concentration (M)
log (cell number per ml)
7
6
5
0
10
20
time (hr)
0
0.1
0.2
0.3
0.4
0.5

Fig. 4C

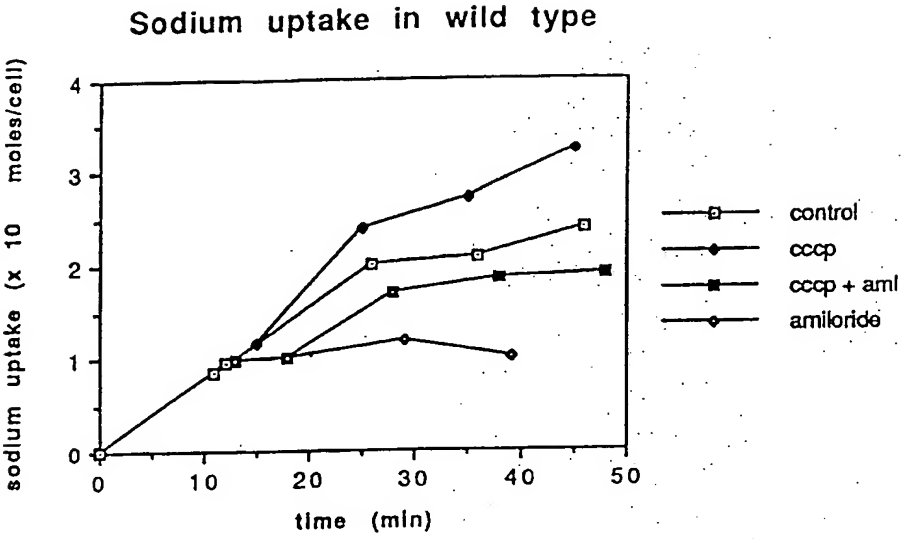
Sodium uptake in wild type
sodium uptake (x 10 moles/cell)
4
3
2
1
0
0
10
20
30
40
50
time (min)
control
cccp
cccp + aml
amiloride

Fig. 5

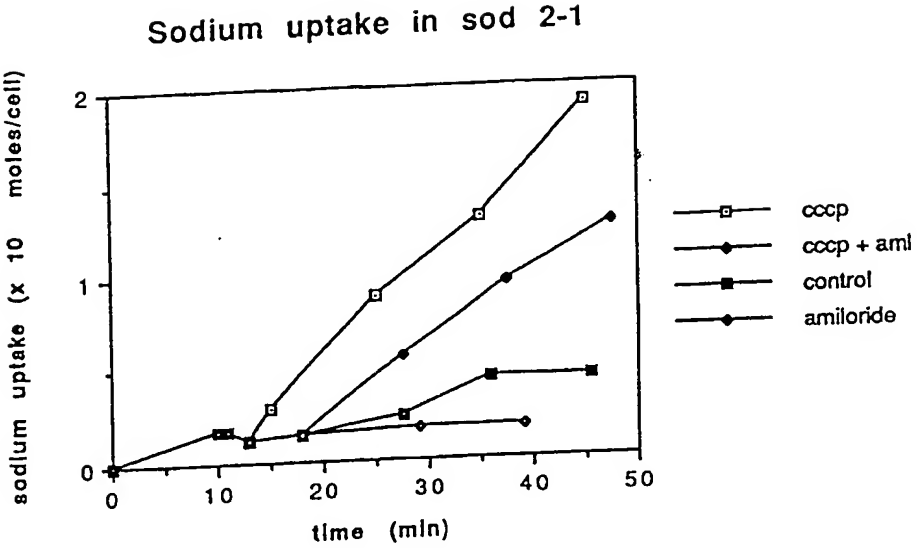
Sodium uptake in sod 2-1
sodium uptake (x 10 moles/cell)
2
1
0
0
10
20
30
40
50
time (min)
cccp
cccp + aml
control
amiloride

Fig. 6

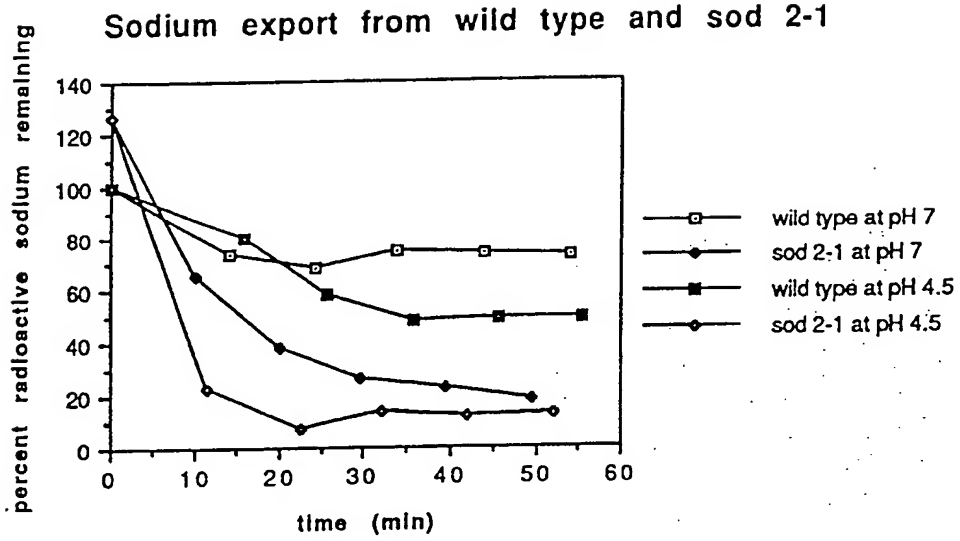
Sodium export from wild type and sod 2-1
percent radioactive sodium remaining
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
time (min)
wild type at pH 7
sod 2-1 at pH 7
wild type at pH 4.5
sod 2-1 at pH 4.5

Fig. 7

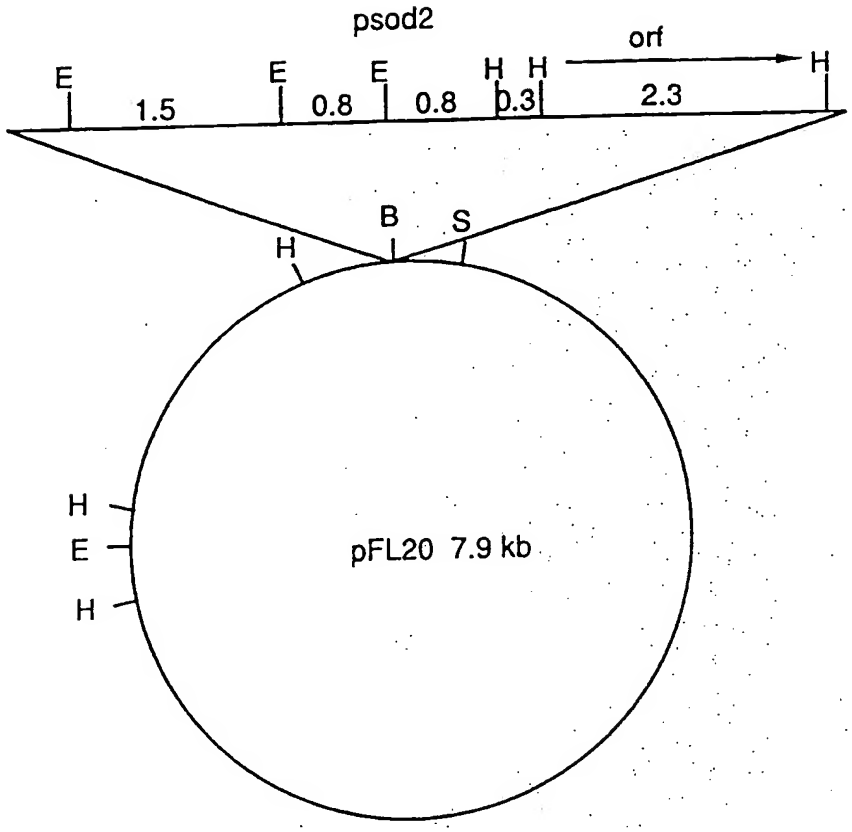

Fig. 8

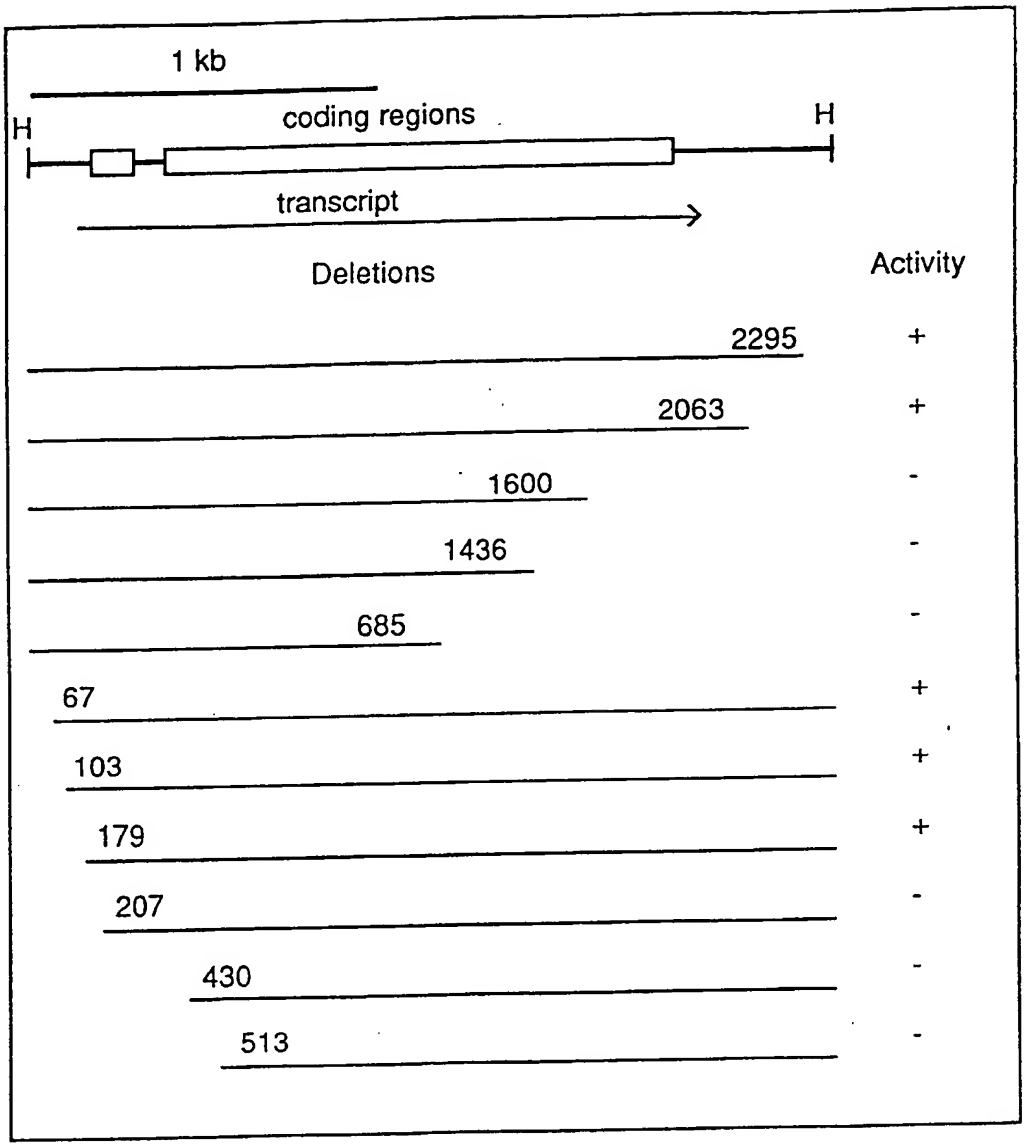
1 kb
H
coding regions
H
transcript
Deletions
Activity
2295
+
2063
+
1600
-
1436
-
685
-
67
+
103
+
179
+
207
-
430
-
513
-

Fig. 9

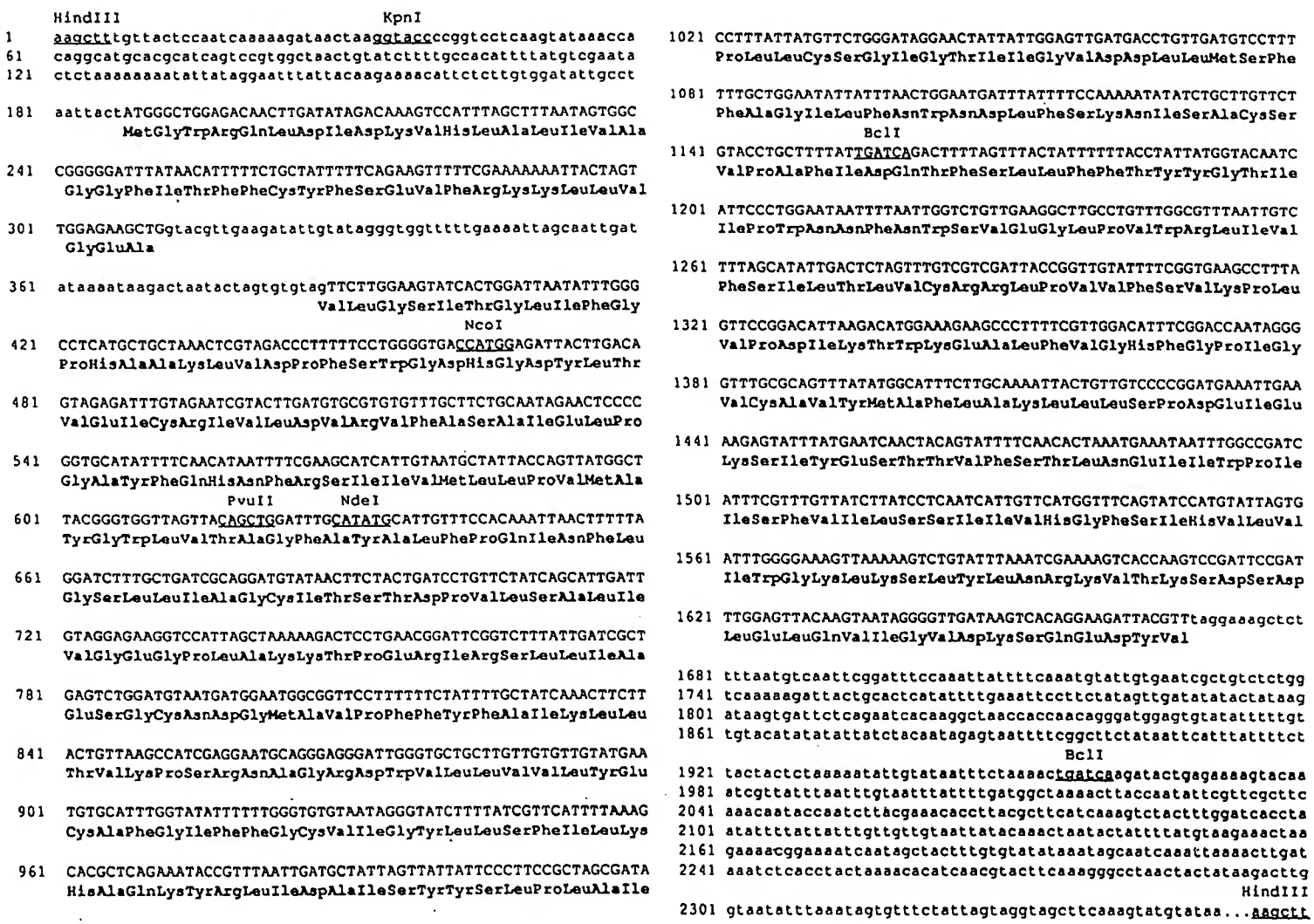

Fig. 10

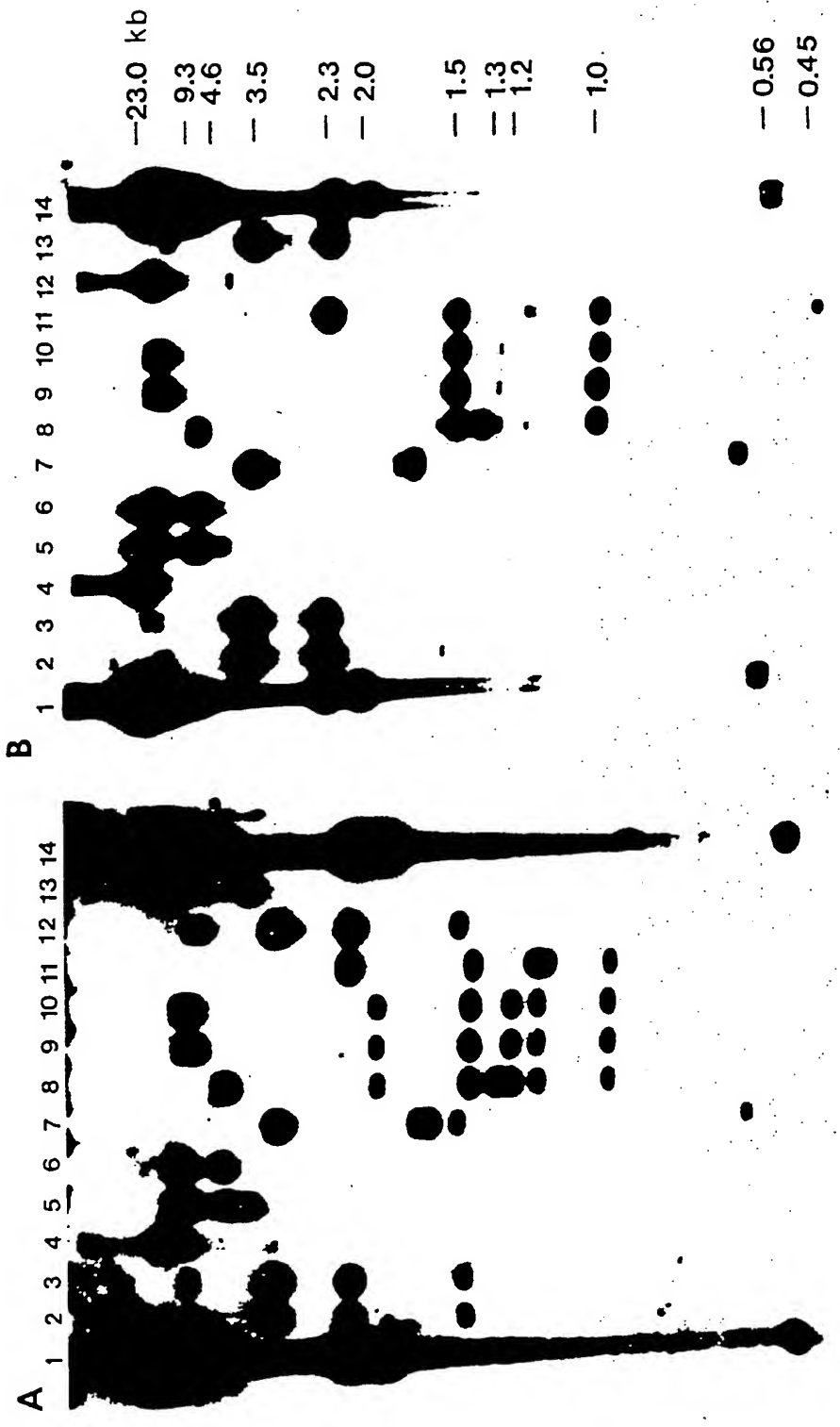
23.0 kb
9.3
4.6
3.5
2.3
2.0
1.5
1.3
1.2
1.0
0.56
0.45
B
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 11A

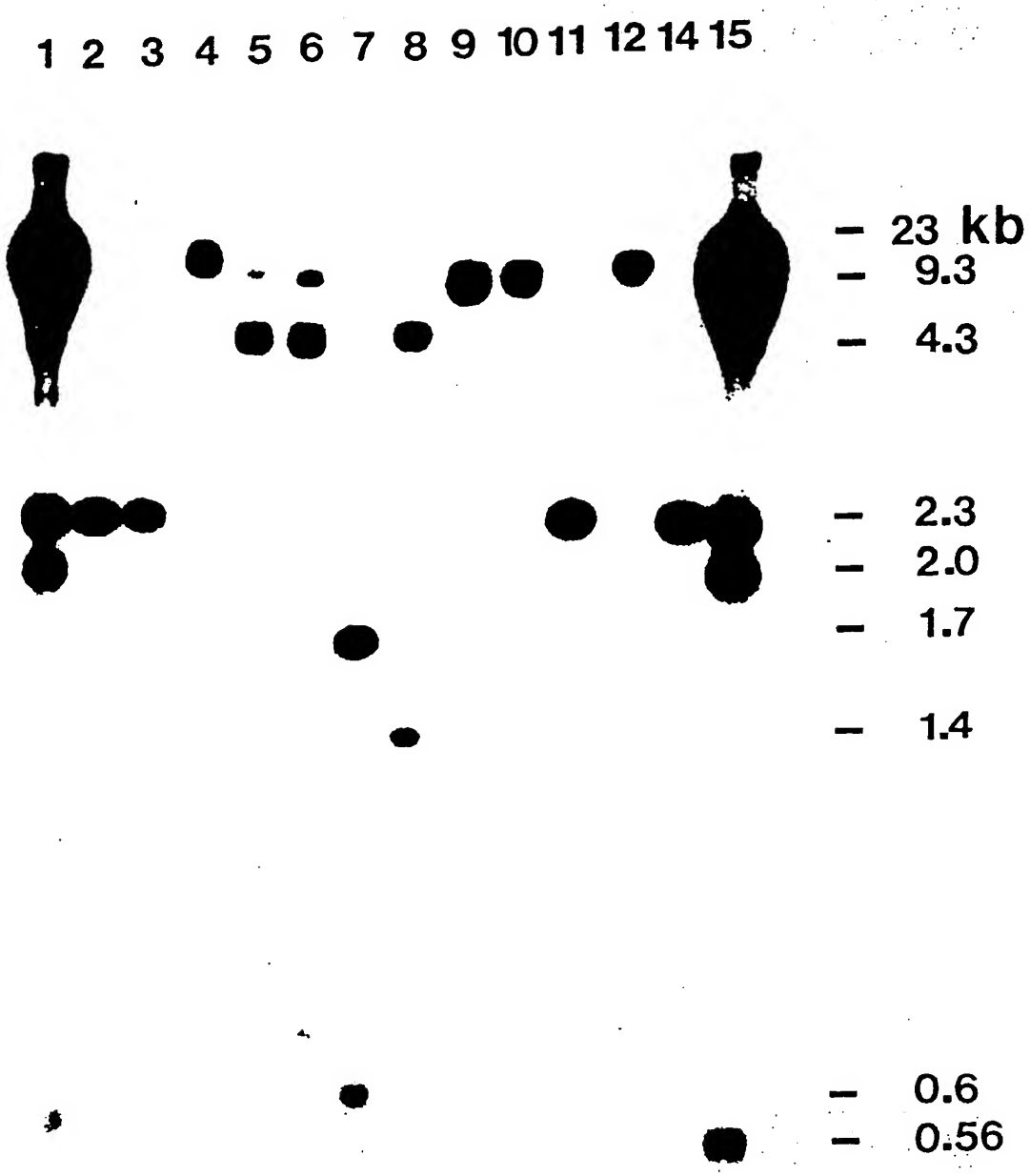

Fig. 11B

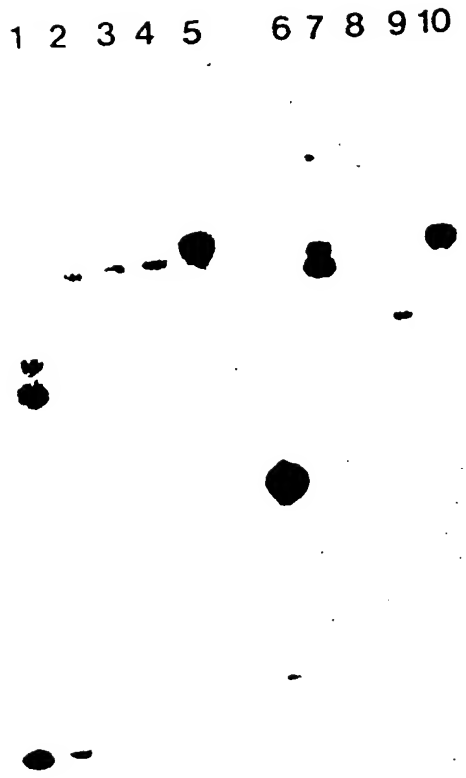

Fig. 12

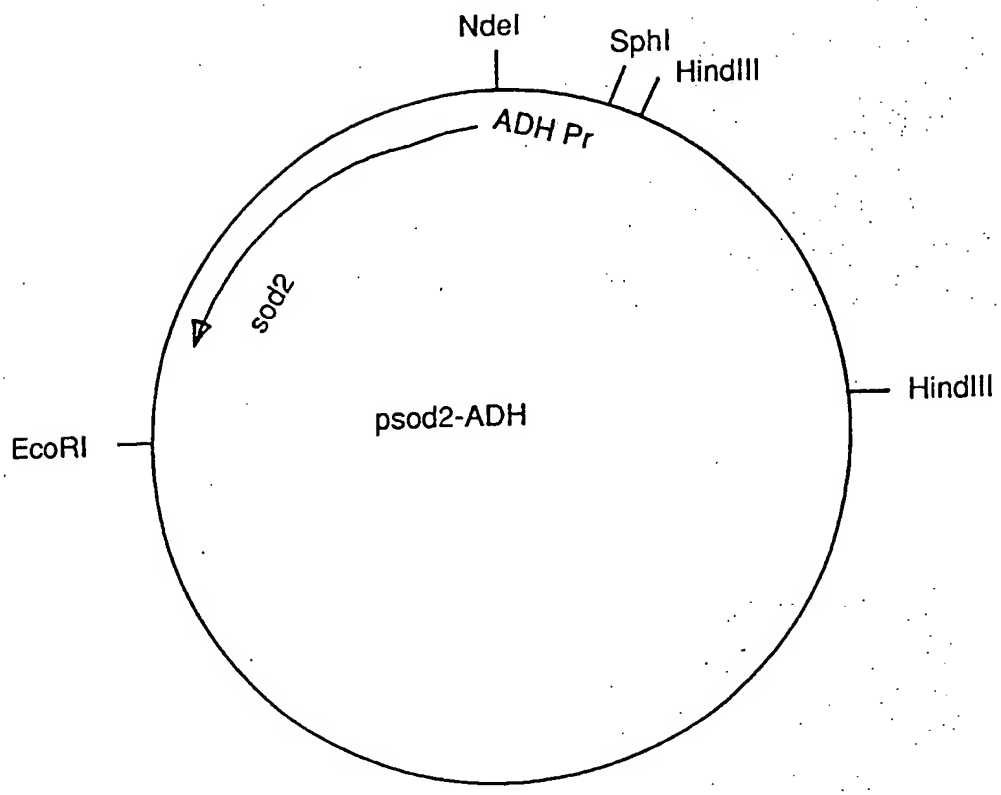

Fig. 13

Lithium tolerance of wild type, sod 2-1, ADH 1 and ADH 2

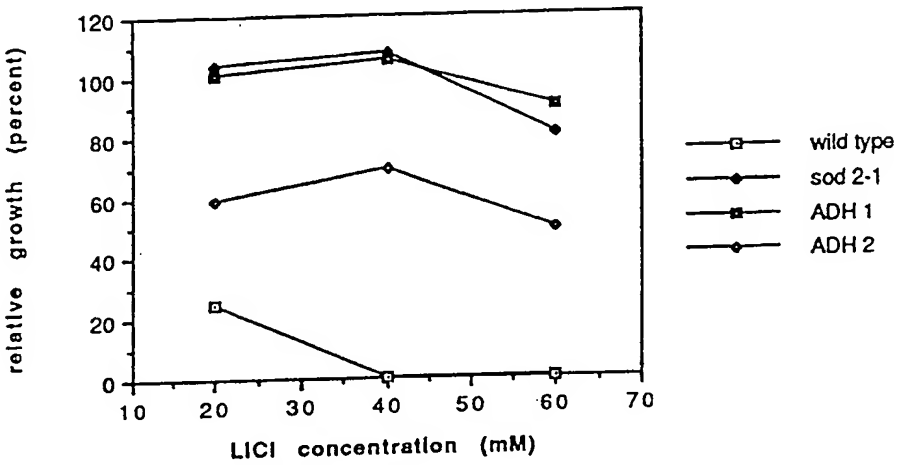

Fig. 14

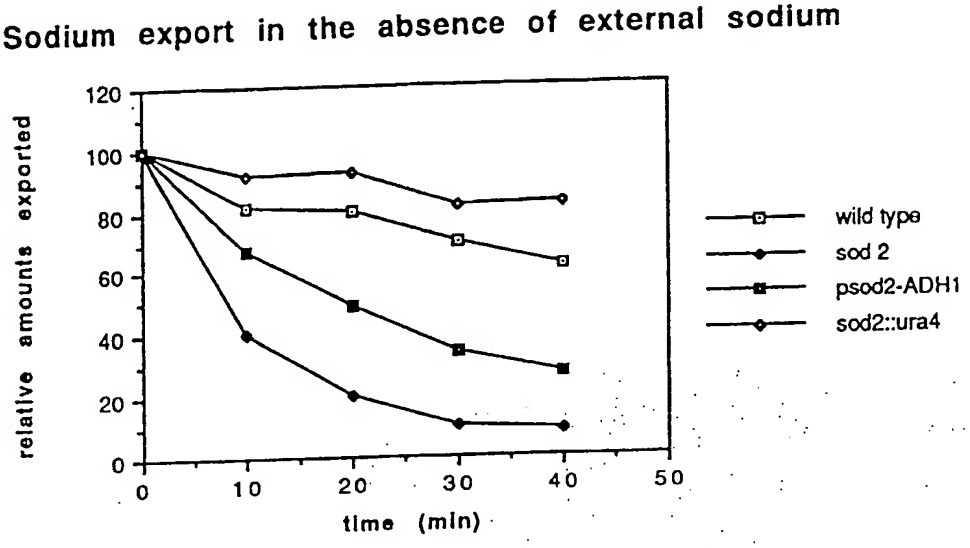
Sodium export in the absence of external sodium
relative amounts exported
120
100
80
60
40
20
0
0
10
20
30
40
50
time (min)
wild type
sod 2
psod2-ADH1
sod2::ura4

Fig. 15

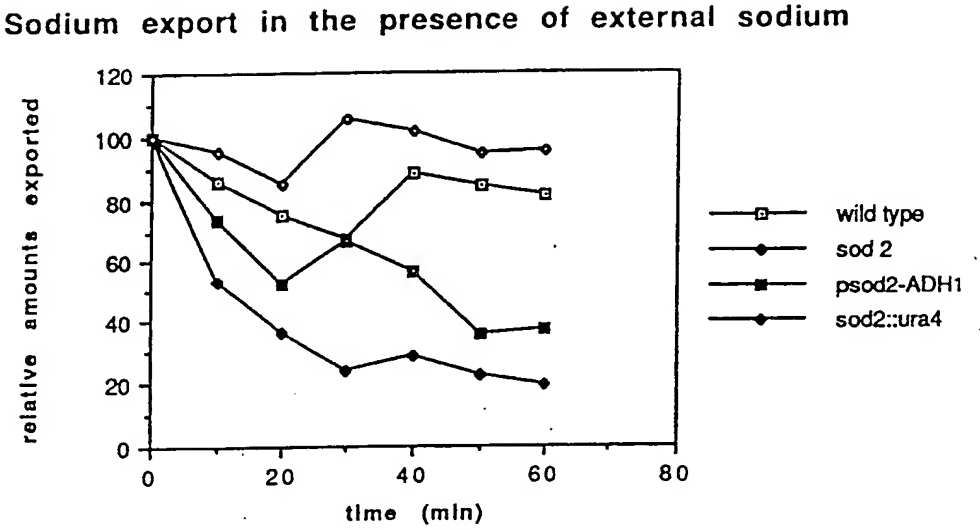
Sodium export in the presence of external sodium
relative amounts exported
120
100
80
60
40
20
0
0
20
40
60
80
time (min)
wild type
sod 2
psod2-ADH1
sod2::ura4

Fig. 16

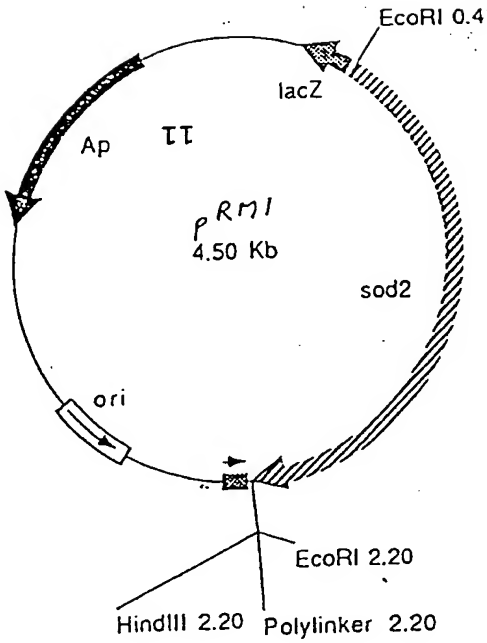
EcoRI 0.41
lacZ
Ap
pRM1
4.50 Kb
sod2
ori
EcoRI 2.20
HindIII 2.20
Polylinker 2.20

Fig. 17

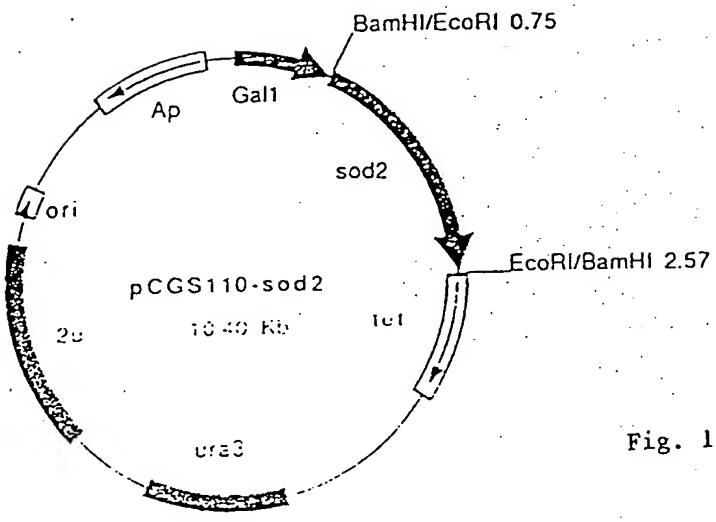
BamHI/EcoRI 0.75
Gal1
Ap
sod2
ori
EcoRI/BamHI 2.57
pCGS110-sod2
ura3

Fig. 18

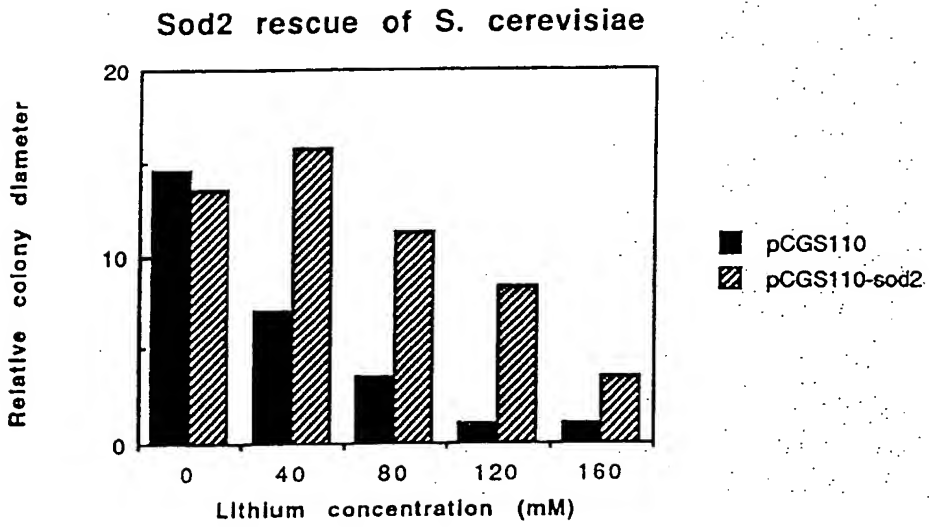
Sod2 rescue of S. cerevisiae
Relative colony diameter
20
10
0
0
40
80
120
160
Lithium concentration (mM)
pCGS110
pCGS110-sod2

Fig. 19

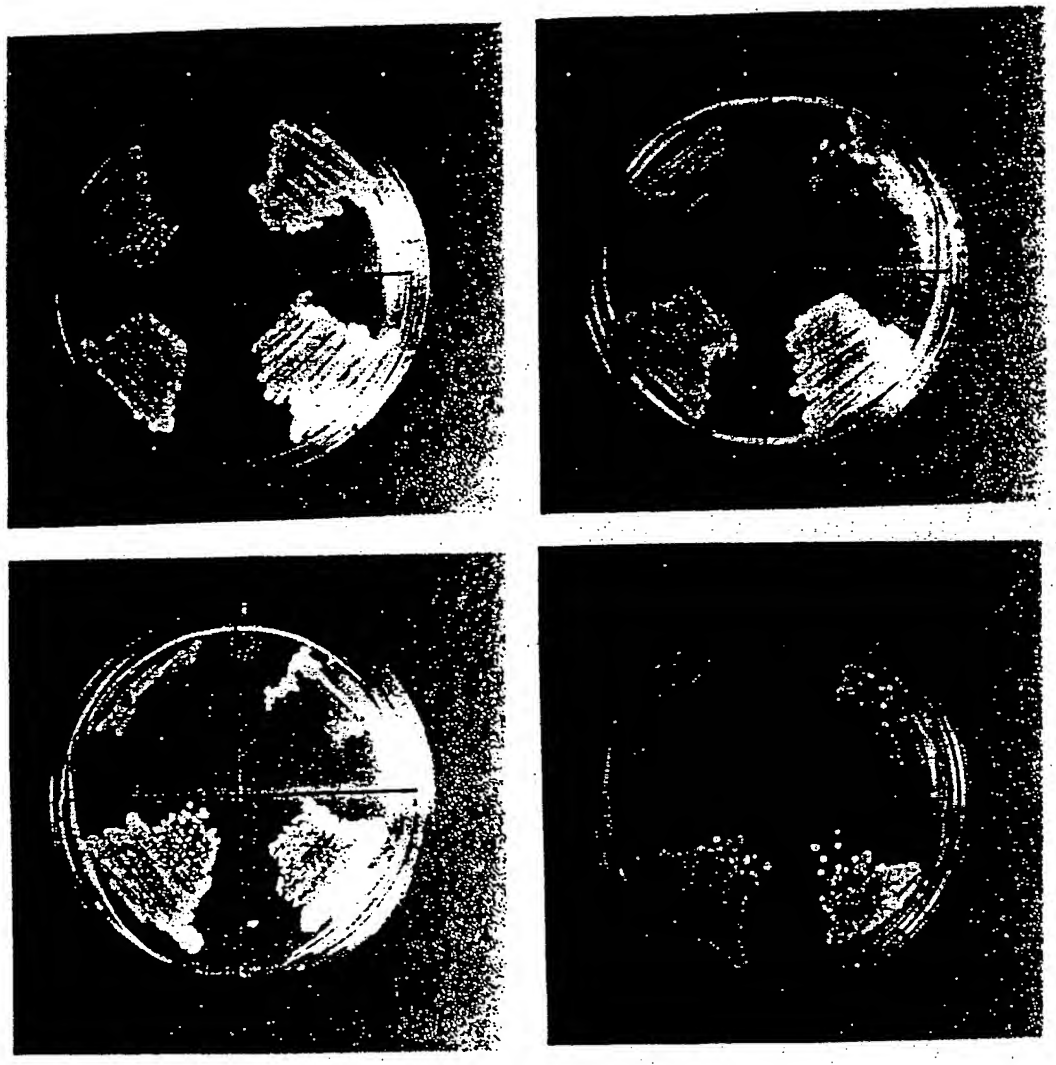

Fig. 20

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 90/00367

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/31, C 07 K 13/00, C 12 N 15/81, C 12 N 15/82, C 12 N 5/10, A 01 H 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N, C 07 K, A 01 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	The Journal of Biological Chemistry, volume 263, no. 21, 25 July 1988, The American Society for Biochemistry and Molecular Biology, Inc., (US), R. Karpel et al.: "Sequencing of the gene ant which affects the Na^+/H^+ antiporter activity in Escherichia coli", pages 10408-10414 see the whole article --	1,3-5,7,9, 17,27,28
X	Chemical Abstracts, volume 108, 1988, (Columbus, Ohio, US), Fan Jianbing et al.: "Cloning and characterization of the yeast Pro2 gene coding for γ-glutamyl phosphate reductase and its enhancing effect on salt tolerance and osmoregulation", see page 201, abstract 181371p & Kexue Tongbao (Foreign Lang. Ed.) 1987, 32(23), 1647-8 --	1,3-5,7,9-11,17,27,28

./.

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16th January 1991	21.02.91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. Pegg

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	US, A, 4594323 (CSONKA) 10 June 1986 see column 3, lines 18-47; columns 7-9	1,3-5,7,9, 17,27,28
A		10-12,15,16, 19,20,22-26
	--	
X	Chemical Abstracts, volume 112, 1990, (Columbus, Ohio, US), K.S. Prakash et al.: "Transfer of saline tolerance from one strain of rice to another by injection of DNA", see page 150, abstract 17284z & Curr. Sci. 1989, 58(17), 991-3	19,22,23,25
	--	
A	Plant Physiol., volume 89, 1989, J. Garbarino et al.: "Rapid induction of Na^+/H^+ exchange activity in barley root tonoplast", pages 1-4 see the whole article	23,25,19
	--	
A	Chemical Abstracts, volume 110, 1989, (Columbus, Ohio, US), J.S. Lee et al.: "Characterization of fusant from protoplast fusion between Saccharomyces cerevisine D-71 and Zygosaccharomyces rouxii SR-S", see page 597, abstract 6333x & Sanop Misaengmul Hakhoechi 1988, 16(4), 297-302	20,22
	--	
A	Biological Abstracts, volume 84, 1987, (Columbus, Ohio, US), E. Blumwald et al.: "Salt tolerance in suspension cultures of sugar beet: Induction of sodium/proton antiport activity at the tonoplast by growth in salt", see page AB-1023, abstract 40333 & Plant Physiol. (Bethesda) 83(4): 884-887. 1987.	23,25,19
	--	
A	Biological Abstracts, volume 88, 1989 (Columbus, Ohio, US), M.D. Ortega: "Potsodium and sodium distribution la vacuole and cyto-plasm of Saccharomyces cerrevisiae", ./.	20,22

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	see page AB-892, abstract 19705 Microbiologia (Madr) 4(1): 61-64. 1988	
	--	
X	Proc. Natl. Acad. Sci. USA, volume 84, May 1987, E.B. Goldberg et al.: "Characterization of a Na^+/H^+ antiporter gene of Escherichia coli", pages 2615-2619 see the whole article	1,3-5,7,9, 17,27,28

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. CA 9000367 SA 40947

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 11/02/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4594323	10-06-86	None	

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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